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GLYCINE TOLERANCE IN EXPERIMENTAL SHALE OIL LIVER NECROSIS.

CYRIL POLSON.

From the Departments of Pathology of the University and St. James's Hospital, Leeds.

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IN a previous investigation (Polson, 1933) seven of the modern tests of liver damage were examined, and of these the galactose tolerance test proved the best. Nevertheless, it failed to detect liver damage on all occasions. It was also shown that estimation of amino-acid nitrogen in isolated samples of blood was valueless except in the terminal phase of experimental liver necrosis. The effects of administering an amino-acid upon the amino-acid nitrogen of the blood are now described. The present glycine tolerance test proved a better method of detecting liver-cell damage than any of the other tests already examined.

v. Falkenhausen (1924) administered 20 gm. of an amino-acid mixture orally to healthy and diseased subjects. The blood amino-acid nitrogen of healthy subjects was estimated in samples taken before and at half-hourly intervals until the end of 2 hours, while observations on diseased persons were concluded at 1 hour after dosage. The maximum rise occurred at ½ hour, and this expressed as a percentage of the initial level was greater in those who had liver disease unaccompanied by jaundice than in healthy subjects and in patients who did not have liver damage. These differences, however, were exaggerated by the method of expressing them. The maximum increase in healthy subjects and .n the patients without liver damage ranged from 1·9 mgm. to 3·4 mgm.; in those with liver disease unaccompanied by jaundice the rise was from 2·2 to 4·3 mgm. Of the latter six, however, the maximum increase exceeded the upper limits of the control groups in three alone. The results do not convince and their value is but apparent as was shown by Witts (1928). Witts administered glycine orally in doses of from 12·5 to 50 gm. to healthy and diseased subjects. Blood samples were taken before and at intervals of 1 and 2 hours afterwards until the end of 6 to 8 hours. The various blood constituents were estimated in these samples. The results, in respect of changes in the amino-acid nitrogen, were interpreted by an examination of the maximum post-absorptive level. This occurred at

1 to 4 hours, on the average at 150 minutes after dosage. Witts found this level higher in the absence than in the presence of liver damage. Although the duration of his experiments permitted observation of a delayed return to the initial level, he ignored this aspect of his amino-nitrogen curves. It is now shown that failure to return to the initial level within a given period is evidence of liver-cell damage.

Wiechmann and Dominick (1926) adopted the present method of interpreting glycine tolerance curves when examining healthy and diabetic subjects. They administered 2·25 gm. of glycine intravenously, and took blood samples before and at short intervals afterwards until the end of 2 hours. They considered that their results showed a delayed return to the initial amino-acid nitrogen level in diabetics. If it be accepted that amounts of less than 0·5 mgm. of amino-acid nitrogen have significance, these authors were justified in their conclusions. If such differences be discounted, an examination of their tables shows that the results were inconclusive. In 8 of 12 healthy subjects the return to the initial level required from 25 to 35 minutes, while in 2 it was not attained until the end of 45 minutes. The return to the initial level was delayed for over 45 minutes in 4 of 10 diabetics, but only prolonged for over 55 minutes in 2. They did not apply the tests to patients with liver disease.

EXPERIMENTAL PROCEDURE.

A glycine tolerance test was devised and applied to 40 healthy rabbits which weighed from 1300 gm. to 2500 gm., averaging 2000 gm. Fifteen of these rabbits were then given shale oil and re-tested daily until killed three or four days later. Liver necrosis, the features of which were previously described (Polson, 1931), was present in all.

Moderate or mild renal damage, shown by observations on the blood and urine, was a concomitant lesion in a proportion of rabbits given shale oil. The glycine tolerance test was therefore applied to 6 rabbits which had severe tubular nephritis following the administration of sodium oxalate. The features of this renal lesion were described by Dunn, Howarth and Jones in 1924.

The method of Folin (1922) was used to estimate the amino-acid nitrogen content of the blood samples. Duplicate estimations showed that amounts of less than 0·5 mgm. had no significance.

All rabbits were killed at the conclusion of the last test, on the third or fourth day of experiment. A post-mortem was at once made, and portions of liver and kidney were submitted to histological examination.

EXPERIMENTAL OBSERVATIONS.

1. *Glycine Tolerance in Healthy Rabbits.*

Doses of 2 gm., 1 gm., 0·75 gm. and 0·5 gm. of glycine (British Drug Houses, Ltd.) were used in the preliminary experiments. Samples of blood, each of 2 c.c. to 3 c.c., were taken before and at ½ hour and at hourly intervals until the end of 4 hours. The amino-acid nitrogen of the blood was raised for a time, but eventually returned to the initial level. A dose of 2 gm.

appeared to damage the kidney, for the bladder urine contained a distinct haze of albumen and casts of epithelial type were seen. The blood-urea was raised to 83 mgm. per 100 c.c. The smaller doses were apparently innocuous, and after the administration of 0·5 gm. there was a return to the initial level of blood amino-acid nitrogen within 3 hours. This dose was selected for the test, and administered intravenously in the form of 5 c.c. of a 10 per cent. aqueous solution. The preliminary experiments also showed that the essential blood samples were those taken before and at 3 hours after glycine. The remainder, with the exception of that taken at 1 hour, were discontinued, since they provided no additional information.

Forty healthy rabbits were submitted to this tolerance test before receiving their food. The initial amino-acid nitrogen level ranged from 6·3 mgm. to 11·8 mgm. per 100 c.c. The increase at an hour was variable and ranged from 0·3 mgm. to 4·9 mgm., and averaged 1·4 mgm. A return to the initial level occurred within 3 hours in 26 of the rabbits. In the other 14 there was a fall below that level by 0·5 mgm. to 1·8 mgm. at the end of 3 hours. The averaged results of the tests showed that the initial level of 8·9 mgm. was raised to 10·3 mgm. at 1 hour, falling to 8·5 mgm. per 100 c.c. at 3 hours after dosage.

2. *The Glycine Tolerance Tests on Rabbits which had Acute Shale Oil Liver Necrosis.*

Fifteen rabbits were tested before and daily after the intraperitoneal administration of 10 c.c. of the lubricating oil fraction of shale oil. Histological examination demonstrated that the severity of the lesion was approximately similar in 10 of these rabbits, and the results of their experiments are alone considered. (Table I.)

When healthy they reacted to glycine in the normal manner as described above. On the first day of experiment the blood amino-acid nitrogen of 3 rabbits (507, 496, and 506) was still raised, by 1 mgm., 1·3 mgm. and 1·1 mgm. respectively, above the initial level at 3 hours after glycine. It appears that there was slight impairment of glycine tolerance in another 4. There was a fall by 0·7 mgm. to 1 mgm. at the third hour in rabbits 531, 523, 529 and 488 when healthy, whereas on the first day of experiment there was but a fall of 0·4 mgm. in rabbit 531, and a return to the initial level in the other 3. As isolated tests, however, the results with these 4 on day 1 were within normal limits.

On the second day of experiment the blood amino-acid nitrogen was raised by amounts of from 0·5 mgm. to 1·2 mgm. at the third hour in 9 of the 10 rabbits. A normal response was observed in rabbit 513. When the consecutive tests on this rabbit were examined, however, there was apparent defect in glycine tolerance. The fall at the third hour when healthy was by 0·8 mgm.; a similar fall was observed on day 1, but no fall occurred on day 2.

Eight of the rabbits, including rabbit 513, were tested on the third day, and each showed a failure to return to the initial blood amino-acid nitrogen level at the third hour. The excess in individual rabbits was greater than that observed on the previous day, and ranged from 1 to 4 mgm. The anomalous results obtained previously from rabbit 513 are considered due to

TABLE I.—*Glycine Tolerance Tests on Rabbits which had Acute Shale Oil Liver Necrosis.*

Rabbit.	Sex.	Weight.	Day of experiment.	Blood amino-acid nitrogen in mgm. per 100 c.c.			Rise in mgm. at 1 hour.	Rise or fall in mgm. at 3 hours.
				Initial level.	1 hour after 0·5 gm. glycine intravenously.	3 hours after 0·5 gm. glycine intravenously.		
531	..	..	Normal	8·2	8·8	7·4	0·6	−0·8
			1	7·4	10·0	7·0	2·6	−0·4
			2	6·7	7·8	7·2	1·1	+0·5
			3	8·5	10·4	10·0	1·9	+1·5
			4	9·1	14·0	15·6	4·9	+6·5
496	..	..	Normal	7·9	9·1	8·3	1·2	+0·4
			1	9·1	10·0	10·4	0·9	+1·3
			2	7·8	9·3	8·8	1·5	+1·0
			3	11·7	13·4	13·4	1·7	+1·7
			4	11·2	14·8	14·8	3·6	+3·6
523	..	..	Normal	9·9	10·5	9·1	0·6	−0·8
			1	8·2	9·3	8·2	1·1	±0·0
			2	8·8	10·8	9·7	2·0	+0·9
			3	14·8	..	18·8	..	+4·0
528	..	..	Normal	8·2	8·5	8·2	0·3	±0·0
			1	7·8	8·8	8·0	1·0	+0·2
			2	6·8	8·8	7·6	2·0	+0·8
			3	8·2	11·7	10·4	3·5	+2·2
529	..	..	Normal	9·0	10·4	8·0	1·4	−1·0
			1	8·5	9·3	8·5	0·8	±0·0
			2	9·7	12·7	10·8	3·0	+1·1
			3	9·3	11·7	10·8	2·4	+1·5
488	..	..	Normal	8·9	9·5	8·2	0·6	−0·7
			1	7·4	9·3	7·4	1·9	±0·0
			2	7·8	8·5	8·8	0·7	+1·0
			3	7·8	10·0	8·8	2·2	+1·0
507	..	..	Normal	8·5	9·1	8·2	0·6	−0·3
			1	7·8	9·3	8·8	1·5	+1·0
			2	7·8	9·3	8·8	1·5	+1·0
			4	10·0	11·7	11·7	1·7	+1·7
509	..	..	Normal	6·8	7·9	6·5	1·1	−0·3
			1	7·8	8·8	7·8	1·0	±0·0
			2	8·8	10·0	9·3	1·2	+0·5
			3	8·8	9·3	10·0	0·5	+1·2
506	..	..	Normal	7·8	8·2	7·4	0·4	−0·4
			1	8·2	10·0	9·3	1·8	+1·1
			2	8·8	10·0	10·0	1·2	+1·2
513	..	..	Normal	10·4	11·0	9·6	0·6	−0·8
			1	8·8	9·3	8·0	0·5	−0·8
			2	8·8	9·3	8·8	0·5	±0·0
			3	12·2	14·8	13·4	2·6	+1·2
Averages	..	..	Normal	8·6	9·3	8·1	0·7	−0·5
,,			1	8·1	9·4	8·3	1·3	+0·2
,,			2	8·2	9·7	9·0	1·5	+0·8
,,			3	10·2	11·6	12·0	1·4	+1·8
,,			4	10·1	13·5	14·0	3·4	+3·9

delayed absorption of shale oil, for it now had a third-hour excess of blood amino-acid nitrogen of 1·2 mgm.

Three rabbits were tested on the fourth day of experiment, and their failure to return to the initial amino-acid nitrogen level at the third hour was even more notable. The excess at the third hour in rabbits 531, 496 and 507 was 6·5, 3·6 and 1·7 mgm. respectively.

When the consecutive tests on individual rabbits were examined, a progressive impairment of glycine tolerance was demonstrated. This is best illustrated by rabbit 531. When healthy there was a third-hour fall by 0·8 mgm., reduced to one of 0·4 mgm. (within the limits of experimental error and regarded as a return to the initial level) on day 1. On the second, third and fourth days there was a failure to return to the initial level at the end of 3 hours, and the excess was by 0·5 mgm., 1·5 mgm. and 6·5 mgm. respectively. The only departure from this progressive impairment of glycine tolerance was observed in rabbit 496. The third-hour excess on day 1 exceeded that of day 2, but there was further increase on the third and fourth days of experiment. This change in the response to glycine was paralleled by progressive histological change, and thus the tests permitted an approximate estimate of the severity of liver damage.

On the fourth day of experiment it was found that the third-hour level of amino-acid nitrogen in the blood of rabbit 531 exceeded that at 1 hour. This was occasionally observed as a terminal phenomenon.

Observation of the blood samples taken at 1 hour after glycine had shown considerable variation in healthy rabbits. Neither was there any uniformity in similar samples from those with liver damage. When averaged, the results from experimental rabbits differed in no way from those of healthy rabbits, except in the terminal phase of liver necrosis. It was therefore sufficient to limit the samples to those taken before and at 3 hours after administration of glycine. Liver damage was indicated by failure to return to within 0·5 mgm. of the initial level of blood amino-acid nitrogen at the end of 3 hours.

3. *Glycine Tolerance Tests on Rabbits which had Oxalate Nephritis.*

Slight damage of the first convoluted renal tubules was occasionally observed in rabbits given shale oil. A separate series of experiments, as yet unpublished. demonstrated renal dysfunction in a proportion of rabbits given shale oil, Renal dysfunction, however, was but moderate, and there was no evidence that the excretion of amino-acid nitrogen was appreciably impaired. In order to demonstrate that impairment of glycine tolerance was not due to concomitant renal damage, the test was applied to 6 rabbits which had oxalate nephritis (Table II.) These had severe damage of the first convoluted tubules, and daily estimation of the blood-urea content by the method of Fowweather (1925) indicated its severity during experiment. Four rabbits had a dose of 50 mgm. per kilo, and 2, 40 mgm. per kilo of sodium oxalate intravenously. The tests on 2 rabbits given the larger dose indicated an impaired glycine tolerance. Histological examination showed that there was a focal liver necrosis, simulating that seen in man in rabbit 573. In rabbit 594 liver damage was also present and was of diffuse type. Fragmentation of the

TABLE II.—*Glycine Tolerance Tests on Rabbits which had Oxalate Nephritis.*

Rabbit.	Dose of sodium oxalate in mgm. per kg.	Day of experiment.	Blood-urea content in mgm. per 100 c.c.	Blood amino-acid nitrogen in mgm. per 100 c.c.			State of liver at death.
				Fasting.	1 hour after glycine.	3 hours after glycine.	
537	40	Normal	38	8·5	9·7	7·9	Healthy.
		2	182	8·1	..	8·1	
		3	173	7·9	..	7·9	
540	40	Normal	40	9·7	11·2	9·3	"
		1	135	11·4	13·6	11·6	
		2	137	9·7	12·1	10·0	
		3	167	10·4	11·4	10·0	
572	50	Normal	28	8·9	11·0	8·9	"
		1	107	7·2	9·1	7·2	
		3	199	9·2	12·1	9·1	
		4	210	7·6	8·4	7·6	
573	50	Normal	43	9·2	10·8	9·1	Focal necrosis.
		1	128	7·4	9·8	9·1	
		3	274	8·8	..	10·1	
574	50	Normal	49	9·1	10·0	8·9	Diffuse moderate necrosis.
		1	210	7·7	10·9	8·7	
		2	274	7·7	11·4	8·7	
575	50	Normal	49	9·7	10·2	9·1	Diffuse mild necrosis.
		1	210	7·0	9·2	6·8	
		2	245	7·8	8·6	7·7	
		3	297	8·9	11·6	10·0	

cytoplasm and pyknotic changes in the nuclei were general throughout the section. The changes were not unlike those observed in the livers of rabbits 2 days after administration of shale oil. Glycine tolerance was not impaired in rabbit 575 until the third day of experiment. It then had slight diffuse damage of its liver. There was a normal response in the tests on the other 3 rabbits throughout the experiments. Severe tubular nephritis was present and liver damage absent in each.

DISCUSSION.

Although the amino-acid nitrogen of the blood was appreciably raised by 0·5 gm. of glycine, administered intravenously, there was a return to the initial level within 3 hours in healthy rabbits. A failure to return to the initial level within 3 hours was observed in rabbits given shale oil as early as the first day of experiment, and this response indicated liver damage.

Unlike galactose tolerance, glycine tolerance was invariably impaired in the presence of liver damage, and when impaired liver damage was present. The glycine tolerance test was the only test yet examined by which an approximate estimate of liver-cell damage was possible. It appeared a better method than the rest for detecting moderate damage. Thus 9 of 10 rabbits had impaired glycine tolerance on the second day of experiment, whereas the galactose tolerance test demonstrated abnormality in only 3 of 5 rabbits on that day.

Mann (1927) criticized the value of experiments of the present kind, since it is impossible to restrict the damage to the particular organ under examination. He drew attention to the probable concomitant renal damage. The response

to glycine by rabbits which had severe tubular nephritis demonstrated that whatever renal damage those with liver necrosis may have had, it was but little responsible for the impairment of glycine tolerance. When glycine tolerance was impaired in rabbits which had oxalate nephritis there was concomitant liver damage.

Witts (1928) neglected the terminal level of blood amino-acid nitrogen in his curves. Examination of his second and fifth charts showed, however, that he obtained a result similar to those now obtained in rabbits. In the second chart he showed that the blood amino-acid nitrogen of a healthy subject, when fasting, was 6·2 mgm. per 100 c.c. Six hours after oral administration of 25 gm. of glycine the level was 6·5 mgm. per 100 c.c., having risen to a maximum level of 9·5 mgm. at 75 minutes. The fifth chart showed results obtained from a jaundiced woman thought to have cholelithiasis. At operation a hobnail liver cirrhosis was demonstrated. Glycine tolerance was impaired in this patient for the initial blood amino-acid nitrogen level of 6·5 mgm. per 100 c.c., was exceeded by 1·5 mgm. at 6 hours after oral administration of 25 gm. of glycine. Unfortunately this was the only example which permitted a comparison between our results, and it therefore cannot be stated that the present method of detecting liver damage is applicable to human disease. The evidence is sufficient, however, to justify further clinical investigation.

Oral administration of smaller doses of glycine, preferably 12·5 gm., which was but little used by Witts, may be employed. The disadvantage of this route lies in the factor of a varying rate of absorption in different individuals. Larger doses may induce vomiting, as shown by Witts. Intravenous dosage as a routine procedure is inadvisable, not only in view of the toxicity of relatively large doses, but also because the technique is difficult. Wiechmann and Dominick (1926) demonstrated that even when 2·25 gm. were administered intravenously the return to the initial level was rapid, and took as little as 15 minutes. Their results, moreover, showed appreciable variation in healthy subjects.

It is now suggested that the subcutaneous administration be used and blood samples taken before and at a given period afterwards. The time for taking the latter would be that at which a return to the initial level of blood amino-acid nitrogen occurred in healthy subjects, and those who suffered from diseases other than of the liver. The suitable amount of glycine was not determined, but a patient given 0·5 gm. subcutaneously suffered neither local nor general effects.

SUMMARY.

A glycine tolerance test of liver damage was devised. Blood samples were taken before and at 3 hours after intravenous administration of 0·5 gm. of glycine to rabbits. When healthy their blood amino-acid nitrogen returned to the initial level within 3 hours. This return was appreciably delayed in those which had shale oil liver necrosis ; a normal response was obtained from rabbits which had oxalate nephritis when unaccompanied by liver damage. Progressive impairment of glycine tolerance in the consecutive daily tests on rabbits was paralleled by progressive liver-cell damage. The test, therefore, permitted an approximate estimate of the damage. Examination of charts

published by Witts (1928) showed that liver disease in a human subject impaired glycine tolerance in a manner similar to that observed in rabbits which had liver necrosis. The superiority of the present test as a means of detecting liver-cell damage in rabbits and the fact that it may also detect such damage in man justifies further clinical examination. A suitable procedure is suggested.

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AN EPIZOOTIC DISEASE OCCURRING IN A BREEDING STOCK OF MICE: BACTERIOLOGICAL AND EXPERIMENTAL OBSERVATIONS.

T. J. MACKIE, C. E. VAN ROOYEN AND E. GILROY.

From the Bacteriology Department and Institute of Animal Genetics, Edinburgh University.

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IN February, 1932, a fatal epizootic disease made its appearances in a mouse colony which was being used by one of us for genetical experiments. The colony was composed of two unrelated stocks, and the disease was limited first to one of these (" S "), though later it spread to the other (" L "). During the preceding period of 18 months the health of the colony had been good and the mortality low. As will be shown, the disease was not due to any of the common infections of these animals. The actual origin of the epizootic could not be ascertained.

The disease showed in most cases characteristic features. The early manifestations were the usual general signs of illness along with moist lustreless coat, semipurulent conjunctival discharge and occlusion of the palpebral

fissures. In those cases which were not immediately fatal, the most frequent feature of the disease was œdematous swelling of the feet and legs, sometimes confined to one or both hind limbs. Sometimes ulceration of the feet with serous discharge and crusting resulted. This at first suggested a superficial resemblance to the early stage of "infectious ectromelia" as described by Marchal (1930), but gangrene was only very rarely observed. In some instances the ankle-joint became greatly swollen, and at autopsy was found to contain an accumulation of caseous material. Frequently the tail was also œdematous, and in a few cases showed multiple bulbous swellings. In a certain proportion of the affected animals swelling of the legs and feet was absent, but the hind limbs became partially or completely paralysed. Another common feature was enlargement of regional lymph-glands. In a few cases in which the disease assumed a subacute form, the conjunctivitis was followed by keratitis and later destruction of the whole eye. In pregnant females gestation was arrested. In the subacute cases emaciation was pronounced. The condition was fatal in the vast majority of cases, particularly in stock "S", death occurring usually in 10 to 14 days. In a certain proportion of animals the condition assumed a more acute form without local manifestations, death resulting in 1 to 3 days. The epizootic attained a maximum about a month after its first appearance, but persisted in the colony in a less pronounced degree during a period of several months. In 4 months over 400 mice died from the epizootic among a population of 600 to 700 adult animals.

At *autopsy* appearances were variable. In the acute cases the organs showed no characteristic changes on macroscopic or histological examination. In the subacute form the spleen was markedly enlarged, sometimes to three or four times its normal size, and numerous areas of necrosis were observed throughout the pulp. In some cases the liver also showed similar necrotic foci. Enlargement of various lymph-glands, submaxillary, axillary, inguinal and abdominal, was frequently noted. Necrotic lesions were occasionally found in the ribs, spinal column and bones of the legs, involving in certain instances the joints. The paralysis of the hind limbs (noted above) could in some cases be referred to a lesion of the spinal column.

In some of its features, *e. g.* conjunctivitis, glandular and splenic enlargement, focal necrosis of spleen and liver, the disease resembled *B. murisepticus* infection as described by Wayson (1927) in field mice, but, as will be shown, was not due to this organism.

INFECTIVITY.

Under natural conditions the infection seemed to be transmitted by contact, and healthy animals placed in cages along with infected mice developed the disease. The infection could be reproduced experimentally in mice by intracutaneous, subcutaneous or intraperitoneal injection of emulsions of spleen, liver and lymph-glands from the naturally infected animals. Heart-blood in some cases was similarly infective. Mice from various sources which had not previously been in contact with infected stock were used for these experiments. The experimentally infected mice developed the pathological condition which characterized the natural disease and died usually within 10 days.

BACTERIOLOGY.

In the earliest cases examined, cultures were made from the heart-blood, spleen and various other tissues on blood-agar plates incubated aërobically at 37° C. After 36 to 48 hours exceedingly minute discrete transparent colonies less than a millimetre in diameter were observed. Film preparations from these revealed small slender Gram-negative bacilli measuring 1–3μ by 0·3–0·4μ, along with more elongated filaments or threads. The colonies were rounded, smooth, convex, translucent and glistening. There was no hæmolysis, nor any visible change in the blood. In some cases the colonies from the inocula were scanty, and at first only observed with difficulty by the naked eye. Sub-cultures from isolated colonies on blood-agar yielded a similar growth. No growth was obtained on ordinary media.

The same type of bacterium was cultivated from over 60 animals taken at random and at different times from those affected by the epizootic, and was also isolated from animals infected experimentally with tissue emulsions (*vide supra*). The organism could not be recognized microscopically with any certainty in the tissues, exudates, etc., of infected animals even when cultivation revealed its presence in considerable numbers. It could be demonstrated (by cultivation) in heart-blood, spleen, liver, kidneys, lymph-glands, brain, conjunctival exudate, and in the subcutaneous tissue of the œdematous legs.

Further observations showed it to be non-motile and non-sporing. After 24 to 48 hours' growth films from cultures revealed considerable pleomorphism, with many curved or wavy filaments often in tangled masses, and similar to the thread forms of certain types of the "influenza" group. Cultures were obtained under both aërobic and anaërobic conditions. The optimum temperature was 37° C. Growth did not occur below 26° C. The thermal death-point was 53° C. (30 minutes' exposure). Cultures of recently isolated strains were only viable for 2 to 4 days.

It was thought at first that the organism might belong to the "influenza" group, but further and detailed investigation of its cultural requirements showed that, though its growth was promoted by blood, it was still able to flourish in the absence of the "X" and "V" factors necessary for the cultivation of *B. influenzæ*. Serum, however, was essential, and growth could be maintained on a serum-medium (*e. g.* Loeffler's) which had been heated to 120° C. for 30 minutes. Unheated serum promoted growth better than heated serum. Several strains were tested in detail for their growth requirements, and the methods described by Fildes (1923) were used for ascertaining whether they were dependent on the "X" and "V" factors. The best growths were obtained on media containing a large proportion of serum, *e. g.* not less than 75 per cent., and Loeffler's medium provided optimum conditions for both primary cultivation and subcultures. In serum-broth the organism grew well, forming a white flocculent deposit in the container.

Plates of Loeffler's medium were ultimately used for the cultivation of the organism from infected animals, and yielded a more abundant growth than that obtained on blood-agar. The colonies were similar in character to those on blood-agar, but of larger diameter (1 to 2 mm.).

In primary cultures from tissues, elongated filamentous forms were sometimes noted with large oval or fusiform expansions situated centrally, eccentrically or terminally, and in media containing a proportion of serum less than the optimum coarse swollen irregular thread forms were also observed. These corresponded to the atypical morphological forms sometimes found in certain strains of the "influenza" group. In serum-broth cultures kept at 37° C. for 7 days or over, the individual organisms had undergone such disintegration or alteration that in microscopic preparations they became morphologically undefined and could not be recognized as bacterial cells.

Tests for fermentation of carbohydrates by the usual methods were unsatisfactory, and any changes in reaction produced were apparently slight. The utilization of sugars was tested by growing the organism in serum-peptone-water with 1 per cent. of the sugar, and then after 2 days determining the change in pH of the medium (with the necessary controls). In glucose, maltose and mannite media a change in the pH towards acid was noted (*e. g.* from pH 7·2 to pH 6·6). No change was observed in lactose, saccharose or dulcite. There was no indole formation in serum-peptone-water. Hæmolysin production was not demonstrated. While recently isolated strains were unable to grow on medium in the absence of serum, after prolonged artificial culture slight growth could be obtained on ordinary media.

Fourteen strains isolated from separate animals at different times were kept for serological tests, and agglutinating antisera for two of them were prepared by immunizing rabbits. In their agglutination reactions all the strains formed a serologically homogeneous group.

EXPERIMENTAL INFECTION WITH CULTURES.

The disease with its characteristic features (as described above) could be reproduced by intracutaneous, subcutaneous and intraperitoneal injection of cultures and also by instillation of cultures into the conjunctival sac. The incubation period varied from a few hours to 10 days according to the dose and the susceptibility of the animal. Different strains of mice showed apparent differences in resistance, the "S" stock referred to above being more susceptible than "L" or mice of genetically impure stocks. (This genetic aspect of the disease will be dealt with by one of us in a later communication.) Animals of high susceptibility (*e. g.* stock "S") injected with 0·25 c.c. of a 48 hours' serum-bouillon culture developed a rapidly fatal septicæmic form of the infection, dying in 24 to 48 hours after inoculation. Smaller doses, *e. g.* 0·25 c.c. of fluid culture, diluted 1 in 100 were still lethal, but the duration of survival was prolonged (7 to 10 days) and the disease assumed the subacute form. Animals belonging to a more resistant stock, when injected even with large doses, usually showed the subacute type of infection. Thirty strains of the organism were used for these experiments, and all proved pathogenic on experimental inoculation. Attempts to transmit the infection by enteral administration of cultures were unsuccessful. Injection of cultures into the foot produced the disease, with primary local swelling and œdema.

Rats, guinea-pigs, rabbits, pigeons and fowls were resistant to inoculation with cultures.

In a series of filtration experiments the organism proved to be non-filterable,

and filtrates of infected tissues were non-infective. Culture filtrates were also non-toxic.

DISCUSSION AND CONCLUSIONS.

In its initial features the disease resembles in some respects the condition described as "ectromelia", which was also reported in a breeding stock of mice. We have made some limited observations of this latter condition in experimetally inoculated mice, and it differs from the condition we have described in the absence of paralysis and conjunctivitis, and the development of a characteristic dry gangrene of the feet with demarcation from the healthy tissue and spontaneous amputation. "Ectromelia" has been reported by Marchal to be due to a filterable non-cultivable virus which can be preserved in dessicated material. In mice experimentally inoculated with the "ectromelia" virus, we have not demonstrated any visible cultivable bacterium by the methods applicable to the cultivation of the organism described in this paper.

The condition also shows some similarities to *B. murisepticus* infection as described by Wayson. For comparison a number of mice were inoculated with cultures of a standard strain of this organism. Fatal infection resulted in the majority of the animals, death occurring after 4 to 14 days, and the characteristic Gram-positive bacillus could be demonstrated in the heart-blood. Some of these animals showed conjunctivitis with occlusion of the palpebral fissures and some developed paralysis of the hind limbs, but in no case was swelling of the feet or legs observed.

The organism we have described is difficult to classify. While it shows a morphological resemblance to certain types of bacteria belonging to the "influenza" group and requires serum (or serum-proteins) for its growth, it is not dependent on the factors in blood necessary for the growth of *B. influenzæ*. We have compared it carefully with a standard strain of *B. murisepticus*, but its Gram-negative reaction and growth characteristics differentiate it clearly from that species. Though it tends towards a leptothricoid morphology, it cannot be identified in its other biological characters with the recognized *Leptothrix* types.

The ætiological relationship of this organism to the disease described has been fully established; it has been isolated in culture with constancy from a large number of naturally infected animals, and cultures on experimental inoculation have reproduced the same pathological condition; from the animals so infected the organism has been recovered and again proved virulent on passage.

We are indebted to Dr. Fildes, who kindly examined a strain of the organism, with regard to the question of its relationship to the influenza group. We have to thank Miss Marchal for a specimen of the ectromelia virus. One of us (E. G.), while engaged in the work recorded, was in receipt of a grant from the Medical Research Council.

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THE RENAL LESIONS IN HYPERVITAMINOSIS D: OBSERVATIONS ON THE URINARY CALCIUM AND PHOSPHORUS EXCRETION.

J. GOUGH, J. B. DUGUID AND D. R. DAVIES.

From the Department of Pathology, Welsh National School of Medicine, Cardiff.

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RENAL calcification is regarded as one of the commonest features of hypervitaminosis D in the rat. It usually appears as a granular deposit of calcareous material scattered irregularly amongst the renal tubules, chiefly in the medulla. In many cases it is the only appreciable change to be seen, but sometimes an extensive parenchymatous degeneration is also present, which may lead to total destruction of some tubules, and to the formation of scar-like areas of shrinkage in the cortex. This change, which must be familiar to most of those who have worked in this field, and which may conveniently be described as a "nephrosis", gives rise to a permanent and progressive renal lesion (Fig. 1) somewhat resembling human chronic nephritis. The frequent co-existence of this nephrosis with calcification may easily lead to the impression that the two are interdependent, but the following experiments show that this is not the case.

The original purpose of the experiments was to investigate the process of calcification, and to find what effect altering the reaction of the urine would have on its development As the most convenient method of varying the reaction, the administration of acid and alkaline orthophosphates was chosen, especially in view of the fact that we have found (Duguid, Duggan and Gough, 1932) that a high phosphate diet given in conjunction with overdoses of vitamin D is particularly conducive to renal calcification. The relationship of the nephrosis to the administration of these salts became evident as an incidental finding.

Experiment 1.

Two groups of 18 full-grown young rats were given a standard basal diet of bread 70 parts and potatoes 30 parts, mashed together into a paste. To the diet of the first group was added 5 per cent. of acid sodium phosphate, and to that of the second 13·8 per cent. of tri-sodium phosphate, thus giving equivalent P in each. These diets were Ca low (less than 0·05 per cent.) and P high (more than 1·0 per cent.). Unlimited tap-water (about 10 mgm. Ca per litre) was allowed for drinking. Twelve of the rats in each group were given 20,000 M.R.C. units of vitamin D daily by mouth in the form of calciferol in olive oil, the remaining 6 serving as controls. (Three rats from the second group died early in the experiment and were discarded.) During

the experiment the rats were kept in separate cages, and each one was transferred on every third night to a urine-collecting cage, where it remained from 6 p.m. until 9 a.m. on the following morning. The 15-hour specimens of urine were collected in vessels containing liquid paraffin, under which the urine settled so that it was protected from evaporation and atmospheric action. During the 15-hour periods food was withheld in order to obviate contamination

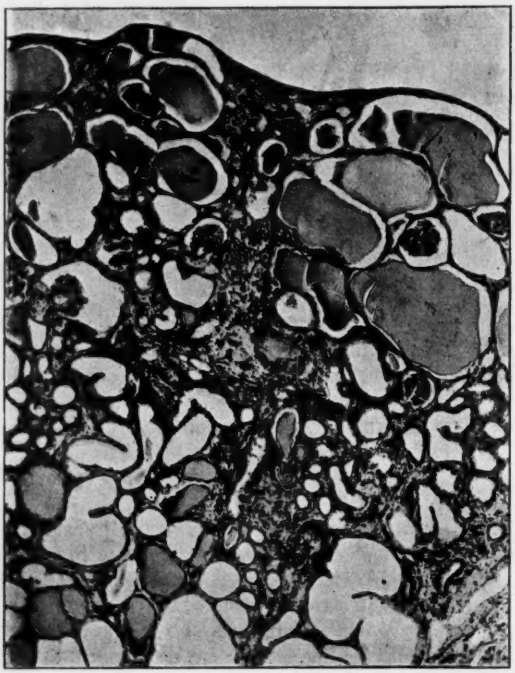

FIG. 1.—Rat kidney. (× 70, hæmalum and eosin.) In parts the tubules are completely destroyed and the connective tissue framework collapsed and condensed. In other parts the tubules are distended, lined by flattened epithelium and, in some cases, filled with "colloid" casts. The capsular surface is very uneven, so that the naked-eye appearance of the kidney is finely granular.

of the urine, and water was supplied in a receptacle so arranged as to make overflow into the urine vessel impossible.

In the absence of calciferol both groups of rats tolerated their salt diets fairly well. Those on the tri-sodium phosphate were at first inclined to reject their heavily salted food, and consequently tended to lose weight, but later they ate more and regained weight. On this account average weight curves do not give a true idea of the toxicity of the two salts. On the whole it appeared that Na_3PO_4, both with and without calciferol, was slightly more injurious than NaH_2PO_4.

The reaction of the urine was measured by the " capillator " method. Before the experiments the urines were round about pH 7·0–7·2. When the animals were placed on the experimental diets those of the rats on the acid diet averaged pH 6·2, whereas those of the rats on the alkaline diets averaged 7·5. Generally the rats receiving calciferol passed slightly more acid urines than their respective controls, although this was by no means a constant feature. The renal excretion of calcium was estimated at intervals in pooled specimens of urine by the Kramer and Tisdall method. No attempt was made to record the amount for each individual rat, since these amounts were usually too small to determine accurately. Thus the urinary calcium figures recorded in the tables represent only the average amount passed per rat per 15 hours. As is seen in Table II, the average was relatively higher in the rats on the alkaline diet than in those on the acid diet, and in either case relatively much higher in the rats receiving calciferol than in the respective controls. The calcium content of the left kidney was also estimated post-mortem in every case. The individual figures (see Table I) varied greatly, but the average of each group generally followed the same course as the urinary calcium figures, the highest being found in the rats on Na_3PO_4 with calciferol. A point of interest is the fact that the renal calcium tended to be higher in the female rats, the average renal calcium in the whole experiment being 7·23 mgm. in females, as compared with 3·17 mgm. in males, thus confirming the observations of Kern, Montgomery and Still (1931).

HISTOLOGY.

The right kidney was examined histologically in every case. Calcification was found in the majority, and always most plentifully where calciferol had been given. Of the two diets the alkaline one contributed by far the most severe degree of calcification, so that in general the histological findings corresponded with the quantitative estimations of calcium in the kidneys. In practically all cases the change involved chiefly a narrow zone at the junction of the cortex and the medulla. In many it was clearly visible to the naked eye on the cut surface of the kidney as a white or yellowish granular line.

Nephrosis, in contrast to calcification, was greatest by far in the rats on the acid diet. It was almost confined to those receiving calciferol with acid sodium phosphate, and no more than a slight trace was seen in any of the others. In the above-mentioned rats it reached a degree of severity far greater than we had ever previously seen it in experimental work with vitamin D. The change, which is to be the subject of a future paper, consisted of extensive necrosis and desquamation, or sometimes shrinkage, of the epithelium with collapse and disappearance of whole groups of tubules, leaving condensed, scar-like areas in the cortex. This was accompanied by irregular dilatation of other tubules, with flattening of their epithelium and hyaline cast formation, so that the histological picture was characterized by alternating areas of shrunken and dilated cortex, which gave to the naked-eye appearance a pronounced granularity. In severe cases the kidney was also pale and swollen with œdema.

TABLE I.—*Degree of Calcification and "Nephrosis" in Kidneys (Exp. 1).*

Rat.	Sex.	Initial weight.	Treatment.	Experiment duration (days).	Renal calcium. Mgms. in whole kidney.	Renal calcium. Mgms. % of kidney.	Histology. Calcification.	Histology. Nephrosis.
474	M.	122	NaH_2PO_4	28	0·40	53	..	..
470	M.	152		39	0·15	18	..	..
469	F.	133		41	0·33	48	+	..
471	F.	100		95	0·53	67	+	+
472	M.	122		95	0·14	18	..	..
473	F.	158		104	1·73	150	+	+
					0·55*	59*		
483	F.	148	NaH_2PO_4	28	1·71	126	+	+++
485	F.	120	with	33	..	..	+++	+++
475	F.	140	calciferol	41	1·73	154	++	++
476	M.	100		45	1·63	118	++	+++
477	F.	123		45	0·61	69	++	++
480	M.	115		62	4·06	447	++	++++
481	F.	121		73	15·15	1073	+++	+++
486	M.	97		77	7·10	715	+++	++++
482	M.	100		81	3·43	331	+++	++++
479	F.	98		86	7·91	767	+++	++++
478	M.	135		88	4·15	350	++	++++
484	M.	123		95	6·32	346	++	+++
					4·89*	409*		
492	F.	130	Na_2PO_4	28	2·28	296	++	..
487	F.	128		40	1·70	187	++	..
488	M.	125		45	1·36	138	++	..
490	F.	112		70	0·61	71	++	..
489	F.	140		94	1·29	141	+	..
491	M.	145		103	1·71	159	+	..
					1·49*	165*		
504	M.	96	Na_2PO_4	28	0·48	64	+	..
503	F.	105	with	40	26·85	1976	++++	+
493	F.	125	calciferol	41	0·72	82	+	..
494	F.	120		45	6·44	761	++++	..
495	M.	143		45	0·70	71	+	+
498	F.	145		63	42·57	3210	++++	..
502	F.	135		72	5·12	515	+++	..
496	F.	110		76	12·87	1095	++	+
497	M.	116		78	12·76	652	++++	..
					12·06*	936*		

* Averages.

TABLE II.—*Average Urinary Excretion of Calcium per Rat, mgms. per 15 hours.*

Treatment.	*1st 6 weeks.	7th week.	8th week.	9th week.	10th week.	11th week.	Whole period.
NaH_2PO_4	0·17	0·37	0·08	0·07	0·16	0·12	0·14
NaH_2PO_4 + calciferol	0·39	0·26	0·56	1·85	0·87	0·80	0·78
Na_2PO_4	0·39	..	0·27	0·12	..	0·40	0·35
Na_2PO_4 + calciferol	0·73	0·82	2·59	3·83	0·75	0·98	1·52

* Up to the end of the 6th week the Ca output in each group remained almost constant.

From this experiment it was clear that whilst renal calcification generally went parallel with the amount of renal excretion of calcium, parenchymatous degeneration was an independent change, related to some totally different factor.

Experiment 2.

In view of the above findings a second experiment was undertaken in an attempt to find what bearing the phosphate metabolism might have on the nephrotic change.

Two groups of twelve rats were given the same standard diets with the respective salts added as in the first experiment. Six from each group were given 20,000 M.R.C. units of calciferol daily by mouth. Fifteen-hour specimens of urine were collected as before, and phosphorus estimations were carried out by titration with uranium acetate. The inorganic phosphorus content of the left kidney was estimated post mortem in each case by the method of Fiske and Subbarow, a portion of the right kidney was taken for histological examination, and the remainder of it was used for an estimation of the total ash content, the amount being too small to serve for a calcium estimation.

The histological findings in this experiment entirely confirmed those of Experiment 1. Similarly, in respect of the calcium content of the kidneys, the total ash estimations bore out the earlier findings.

The records of the urinary phosphorus excretion (see Table IV) varied considerably, and only the average figures for each group need be considered. They were found to be in practically the reverse order of the figures for the urinary calcium, the average phosphorus excretion being relatively high in the rats on the acid diet and low in those on the alkaline, and being considerably lowered in both cases by calciferol. Thus, in comparing the urinary findings in the two experiments, it was noted that circumstances which raised the calcium generally tended to lower the phosphorus excretion, and *vice versâ*. As regards any relationship between the nephrosis and the urinary phosphorus, nothing could be traced, for whilst calciferol lowered the excretion of phosphorus in the rats on the acid diet, it increased the incidence of nephrosis amongst them. The amount of phosphorus found in the kidney post mortem (Table III) did not appear to be relatively influenced by the one or other salt diet alone, but where calciferol had been added the amount in both cases was very notably increased, and especially in the case of the rats on the acid diet, which, it may be noted, were the rats with severe nephrotic lesions. This was the only finding to which the nephrosis appeared in any way referable.

DISCUSSION.

In so far as they are concerned with the incidence of calcification and nephrosis, which are the chief points of this paper, the results seem clear. The two lesions do not necessarily coincide, but appear to be independent of one another. Calcification goes parallel with an excessive urinary excretion of calcium, whilst nephrosis appears to be dependent on some other factor, and cannot safely be attributed to any of the metabolic disturbances recorded.

TABLE III.—*Amount of Inorganic Phosphorus, and Degree of Calcification and Nephrosis in Kidneys (Exp. 2).*

Rat.	Sex.	Initial weight.	Treatment.	Experiment duration (days).	Renal inorganic phosphorus.		Histology.	
					Mgms. in whole kidney.	Mgms. % of kidney.	Calcification.	Nephrosis.
541	M.	243	NaH_2PO_4	19	0·55	48	..	..
542	F.	135		20	0·22	36	..	..
543	M.	158		55	0·50	68	..	..
544	F.	155		82	0·33	44	+	..
545	M.	267		83	0·55	42	+	+
546	M.	222		86	0·52	62	+	..
					0·45*	50*		
547	M.	150	NaH_2PO_4	19	4·57	322	+++	+++
549	F.	130	with	19	3·14	296	++	+++
551	M.	112	calciferol	19	2·08	260	++	+++
550	M.	196		80	3·00	283	++	+++
548	M.	298		82	3·24	154	+++	++++
552	M.	275		82	2·16	114	+++	++++
					3·03*	238*		
553	F.	83	Na_2PO_4	20	0·33	63	+	..
554	M.	225		22	0·49	40	++	..
556	M.	305		73	0·55	39	..	..
555	M.	145		80	0·20	25	+	..
558	M.	183		80	0·57	57	..	..
557	F.	182		81	0·41	49	++	..
					0·43*	46*		
563	M.	88	Na_3PO_4	15	0·82	123	++	..
564	M.	245	with	16	1·44	100	++	..
561	F.	165	calciferol	20	3·89	363	+++	++
559	M.	155		81	0·44	50	++	..
560	M.	250		82	1·00	71	+++	+
562	M.	190		83	0·51	52	+	..
					1·35*	127*		

* Averages.

TABLE IV.—*Average Urinary Excretion of Phosphorus per rat, mgms. per 15 hours.*

Treatment.	1st week.	2nd week.	3rd week.	4th week.	5th week.	6th week.	7th week.	8th week.	Whole period.
NaH_2PO_4	44·5	52·1	33·3	22·6	31·2	20·0	53·8	58·0	39·4
NaH_2PO_4 + calciferol	30·2	22·9	24·6	25·7	30·7	19·4	49·3	43·4	30·8
Na_2PO_4	24·5	27·6	29·0	22·2	19·2	25·9	30·8	25·3	25·6
Na_2PO_4 + calciferol	21·9	18·1	22·4	14·0	14·8	15·0	17·0	15·7	17·4

Although Experiment 2 showed nephrosis to be associated with a high phosphorus content of the kidney, it cannot safely be attributed to this, because kidney disease itself is capable of causing phosphate retention (Greenwald, 1915; Byrom and Kay, 1927, and many others), and the high figure recorded

in the renal tissues may simply have been a reflection of a hyperphosphatæmia due to renal insufficiency. Nor can it be attributed to a high renal excretion of phosphorus, because it did not reach its highest intensity in the group of rats which showed the highest excretion, namely, those receiving acid sodium phosphate without calciferol. Finally, it did not appear to be attributable to acidity of the urine because, when a comparison was made between the urine reaction curve and the degree of nephrosis in individual cases, no parallel could be detected. The only item with which the nephrosis seemed in any degree directly associated was the administration of acid phosphate with excessive vitamin D.

Mono- and di-sodium phosphates have already been recognized as renal poisons, and kidney lesions produced in rabbits by injections of these substances have been described by Hirsch (1923). Nothing has been determined, however, regarding the essential factor in the poison; and the fact that excessive vitamin D exaggerates the toxic effects appears to be a new observation. The latter may eventually prove useful in throwing some light on the problem, but for the present a sufficiently critical interpretation of our results is difficult on account of the limited amount of data obtained. Records of the serum-Ca and P and of the Ca and P balance along with tests of renal sufficiency are required, and these are hardly possible in animals so small as the rat. We hope to be able in the near future to carry out such tests in rabbits.

In addition to the above considerations, there are some points about the findings in regard to the calcium and phosphorus metabolism in these experiments which require to be analyzed. A comparison of the effects of the two diets without calciferol showed that the alkaline one produced a much higher urinary calcium than the acid one. This was unexpected, because acidifying drugs have usually been observed to raise the urinary calcium more than alkaline ones (Givens and Mendel, 1917; Bernhardt, 1924; Stewart and Haldane, 1924). By increasing the intestinal absorption of calcium they tend to shift the calcium excretion mainly to the urine. In our experiment, however, an interfering factor was present in the shape of a very high PO_4 intake, which resulted in a correspondingly high urinary phosphorus excretion, and this latter was especially emphasized in the rats on the acid diet. Acids may have the effect of raising the urinary phosphates (Haldane, Wigglesworth and Woodrow, 1924) as well as the urinary calcium. The significant point in our results was the fact that they did not raise both together. In order to find a possible explanation of the acid lowering instead of raising the urinary calcium, when it raised the urinary phosphorus, reference must be made to what is known of the balance of these elements in the blood-serum. In the serum the Ca and P ions have been shown to exhibit a reciprocal relationship with one another, an excess of one accompanying a diminution of the other (Bourne and Campbell, 1932; Fraser, 1932, and others); thus the administration of orthophosphates has been shown to promote a hypocalcæmia (Binger, 1917). When the figures for urinary Ca and P are compared, it is seen that where P (Exp. 2) is high, Ca (Exp. 1) is relatively low, or *vice versâ*, and it would therefore appear that a similar relationship obtains in the urine. In our experiments phosphorus was the preponderating element, and it may be that, through the action of

this relationship, the lowered calcium excretion was in some way dependent on raised phosphorus excretion.

This tentative hypothesis still holds when the findings in the rats receiving calciferol are examined. In these another antagonizing factor was introduced, for, as is well known, excess of vitamin D is a powerful agent in raising the urinary calcium. It raised it very considerably in both groups of rats in these experiments, but the significant point is that whilst raising the urinary calcium it lowered the urinary phosphorus. It seems difficult, therefore, to avoid the conclusion that the results of these experiments were governed largely by the fact that a reciprocal relationship of the two elements obtains in the animal body.

In view of the recent report by Cramer (1932) of renal lesions, superficially not unlike those with which we are dealing, and produced in rats by a diet deficient in magnesium, we were led to suspect that such a deficiency might be playing a part in our results. Accordingly, an experiment, which need not be described in detail here, was undertaken in which a group of twelve rats receiving acid sodium phosphate with calciferol as in the above experiments, were compared with another group of twelve rats receiving the same diet with an addition of 0·3 per cent. (of the diet) of magnesium sulphate. Both groups of rats were found to develop nephrotic changes about equally, so that the addition of $MgSO_4$ appeared to correct no deficiency of any consequence in relation to this problem. It should be noted, however, that Dr. Cramer has personally demonstrated to us some points of difference between the lesions he found and those which we have been describing.

SUMMARY AND CONCLUSIONS.

The occurrence in the rat's kidney of calcification and of parenchymatous degeneration (" nephrosis ") in hypervitaminosis D were investigated.

The two lesions, which frequently co-exist, were found to be largely independent of one another. Calcification went parallel with a high renal excretion of calcium. Nephrosis could not be clearly correlated with any of the urinary disturbances recorded, but seemed to be in a large measure dependent on the administration of NaH_2PO_4 with overdoses of vitamin D.

The effects of large doses of the acid and alkaline orthophosphates, both with and without hypervitaminosis D (calciferol, 20,000 M.R.C. units daily), were compared.

The urinary excretion of calcium and the deposition of calcium in the kidneys were found to be greatest when the alkaline phosphate was given, and especially when calciferol was added.

The urinary phosphorus excretion was greatest when the acid phosphate was given. Calciferol had the effect of lowering it, both when the acid and when the alkaline salts were given.

The amount of phosphorus found post mortem in the kidney was greatest when calciferol had been given with the acid diet, which were the circumstances under which the most severe nephrosis developed.

The large accumulation of phosphorus in the kidney in the nephrotic rats was thought to be a mark of retention of phosphates caused by the renal disease.

The urinary excretions of Ca and P appeared to show a reciprocal relationship to one another, a relative increase in the one being accompanied by a corresponding lowering of the other.

The expense of this research was borne largely by the Beaverbrook Fund. The calciferol used in the experiments was very kindly supplied to us by Messrs. Glaxo, Ltd., and we are greatly indebted to H. Jephcott and A. L. Bacharach for their kindness in this matter.

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THE O-RECEPTOR COMPLEX OF *B. PROTEUS* X19.

P. BRUCE WHITE.

From the Department of Bacteriology, National Institute for Medical Research, London.

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THE following experiments have been carried out with 4 anti-O X19 (rabbit) sera and 11 samples of typhus serum (X19 type). Of the typhus sera, 7 were received from Dr. I. Balteanu, of Bucharest, while 4 were of Malayan origin, and received from Dr. A. Felix.

It was found that Seitz filtrates of boiled saline suspensions of *B. proteus* X19 caused vigorous precipitation both in anti-O X19 sera and in the sera of typhus sufferers, and that in both cases, to a degree varying with the precipitating potency of the extract, the sera so treated lost their agglutinating action on the bacillus.

By treating mass suspensions of alcohol-washed (or boiled) X19 bacilli with trypsin (2 per cent. at pH 8) at 37° C. for periods varying from 2 to 18 hours, and dissolving in a small volume of saline the precipitate thrown down

from the digestion mixture by 2½ volumes of alcohol, solutions with similar precipitating and agglutinin binding properties, but of greater potency, were obtained. These solutions were heated to 100° C. before use to inactivate any trypsin present.

The agglutinin-absorption experiments were made by diluting the serum 1 : 10 or 1 : 20 with the bacterial extract, and allowing the mixtures to stand for 2 hours at 37° C. and overnight at room or ice-box temperature before filtering off the precipitate by means of a Seitz apparatus. The agglutinating power of this absorbed serum was then compared with that of a sample of the original serum also passed, when similarly diluted with saline, through a Seitz filter. Filtration in itself sometimes occasioned a slight loss of titre; as Seitz K-discs showed less of this absorptive action than the finer EK filters, and were, nevertheless, efficient in removing the precipitates, they were usually employed.

In these tests, performed with materials which had not been exposed to strong, hot alkali, the agglutinins of the rabbit antibacterial sera and of the human typhus sera behaved, to all appearance, in an identical manner. When, however, attempt was made to obtain specific substances from X19 bacilli dissolved at 55° C. in 0·5–1·0 per cent. NaOH solution or 5 per cent. antiformin, a very different result was obtained. The alkali-treated cultures yielded a soluble specific substance which, in precipitation and in agglutinin-absorption tests, reacted intensely with *Typhus exanthematicus* serum, but little or not at all with O X19 sera.

Potent solutions of this substance were obtained as follows: the alcohol-washed growth from 4 tray (11·5 in. × 9·5 in.) cultures of X19 was treated for 2 hours with about 200 c.c. of 1 per cent. NaOH solution (or 5 per cent. antiformin); 3 volumes of alcohol were added to the alkaline mixture, and the precipitate was collected and made up in 50 or 100 c.c. of saline; the solution was brought to pH 7·2–7·4 and clarified by filtration.

Search in the alcoholic supernatant for the missing receptors for anti-X19 sera was without result, and it was found that on treatment with 0·5 per cent. NaOH solution at 55° C. for a few minutes, saline extracts of X19 and the specific substances obtained, as earlier described, from trypsinized cultures, lost almost entirely their power of reacting with X19 antisera, though retaining in full that of reacting with typhus sera.

In Table I certain of the results—typical of all—obtained with these various solutions are set out.

The facts would seem to admit of only one explanation—that the "O", heat-stable, somatic complex of *B. proteus* X19 presents two distinct serological receptors:

A, an alkali-labile receptor, which is overwhelmingly responsible for the O-agglutination of the organism by its own antiserum, but which plays no part in the Weil-Felix reaction with typhus serum.

B, an alkali-stable receptor, which is entirely responsible for the reaction of the bacillus with typhus serum, but which plays only a minor or minimal part in the reaction with X19 serum.

Various efforts have been made to complete the argument by separating, in an active form, the alkali-labile factor A from its more alkali-stable associate,

TABLE I.

Treatment of serum.		Agglutination titre, for proteus O X19 of—	
How treated to absorb agglutinins.	Whether filtered through Seitz K disc.	Anti-O X19 serum (No. 2).	*Typhus exanthematicus* serum (Roumanian 6).
(*a*) Unabsorbed	No	1 : 5000 (+++)	1 : 2500(+++)
(*b*) Unabsorbed	Yes	1 : 2500 (++++), 1 : 5000 (++)	1 : 2500 (++)
(*c*) Absorbed with soluble specific substances precipitated by alcohol from a trypsinized suspension of O X19	,,	<1 : 100	<1 : 100
(*d*) Absorbed with the same specific substances as in (*c*), after these had been treated with 0·5 per cent. NaOH at 55° C.	,,	1 : 2500 (++++), 1 : 5000 (++)	<1 : 100
(*e*) Absorbed with soluble specific substances precipitated by alcohol from a solution of O X19 bacilli in 1 per cent. NaOH	,,	1 : 2500 (++++), 1 : 5000 (+)	<1 : 100

Absorption tests were carried out with sera diluted 1 : 10 and 1 : 20 with the test solutions; there was no appreciable difference in the results obtained in the two series; those stated were obtained with sera diluted 1 : 10.

++++, +++, ++ and + indicate decreasing degrees of agglutination; they show the relative intensity of clumping in the last "positive" tube of each series put up. The readings were taken after the tubes had stood for 4 hours at 50° C. and at room temperature for 3 hours; overnight readings were unaltered.

but up to the present no satisfactory method has been devised. Nevertheless, since the alkali-labile factor often seems to predominate in the specific material collected from trypsin-treated cultures, the effect of the typhus-reacting alkali-stable agent may in such cases be, to some extent, diluted out. Using anti-O X19 and typhus sera of approximately equal titre for X19 and a suitably diluted solution of this material, it has been possible to show selective reaction with anti-O X19 serum.

Some experiments were also made with the X Kingsbury strain and small samples of anti-XK rabbit sera and XK type typhus sera very kindly furnished by Dr. Felix. But in this organism the O-receptors reacting with the anti-bacterial serum, unlike those of X19, showed no particular sensitiveness to alkali, and no means of differentiating them from those reacting with tropical typhus serum was discovered.

The case of *B. proteus* X19 finds a partial parallel among Salmonellas in that of *S. paratyphosus B*, and *S. aertrycke*. Some years ago I pointed out (White, 1926) that the common O-complex of these two types consisted of a more important and distinctive factor I and a lesser factor II, which is shared by, and is dominant in, *S. abortus equi* and the Reading type. Fürth and Landsteiner (1929) noted, and I have confirmed, that the factor I is destroyed by alkali (by N/100 NaOH during 1 hour at room temperature), while factor II is alkali resistant. There is, however, this difference between the case of *S. paratyphosus B* and that of *B. proteus* X19: in the former the alkali-stable factor plays an appreciable part in the antigenicity of the organism; in the latter it apparently does not.

While it cannot be suggested that these observations bring us nearer an explanation of the Weil-Felix reaction, they do indicate that the proteus agglutinins of typhus serum are not homologous with those of an anti-proteus (rabbit) serum.

The scope of the work has been strictly limited by the quantity of typhus serum available at the time, and I have been satisfied to attack the serological problem without attempting a purification of the reactive substances, which would, of necessity, have to be checked at each stage by titrations of potency.

To Dr. I. Balteanu and Dr. A. Felix, to whom I am indebted for all the typhus sera employed, I offer my thanks.

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THE CORTICAL LIPOID OF THE MOUSE SUPRARENAL AFTER UNILATERAL SUPRARENALECTOMY.

RAYMOND WHITEHEAD.

From the Department of Pathology, Victoria University, Manchester.

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COMPARISON of a pair of mouse suprarenals, one removed before, the other after, an experiment, would be the method of choice for the study of cortical lipoid, provided that removal of one suprarenal caused no significant change in the amount of lipoid in its fellow. The use of the method by Wolff (1927) and Deanesly (1931) without adequate test of this point necessitated the present inquiry, which demonstrates that the method is untrustworthy.

MATERIAL AND METHODS.

Male white mice from 125 to 188 days old were used. Oats and water were given freely. The mice were killed by fracture of the neck. The suprarenals were fixed overnight in 10 per cent. formol saline, cut frozen, and stained with Sudan III and hæmalum. At least two sections of each gland were examined.

Surgical Technique.

Urethane-ether anæsthesia was used. The mice were made drowsy by the following freshly-made solution: Urethane 20 gm., sodium chloride 0·85 gm., distilled water to 100 c.c. The dose, urethane 1 mgm. per gm. body weight, was injected subcutaneously at the back of the neck. Ether vapour was given just before making the lumbar incision through which the left suprarenal was removed. Six per cent. of the mice died within one day after the operation. Control laparotomies differed from the suprarenalectomies only in the fact that the left suprarenal was not removed.

Estimation of Cortical Lipoid.

Lipoid Measurements.

The breadth of the total cortex was measured in micrometer units in each suprarenal. The total cortex in adult males is usually composed of (*a*) capsule, (*b*) lipoid-free permanent cortex outside lipoid band, (*c*) lipoid band, (*d*) lipoid-free permanent cortex inside lipoid band, (*e*) perimedullary fibrous band.

In one band the five components, denoted by the letters just used, had the following breadths:

a	*b*	*c*	*d*	*e*
1	2	15	3	0

From these measurements the ratio of the breadth of the lipoid band to the breadth of the permanent cortex is calculated.

Where L denotes " breadth of the lipoid band " and PC denotes " breadth of the permanent cortex "—

$$L/PC = c/(b + c + d) = 15/(2 + 15 + 3) = 0{\cdot}8.$$

Lipoid Density.

The total amount of lipoid in the cortex depends not only on the area of the cortex occupied by lipoid, but also on the size and number of lipoid droplets within this area, *i. e.* on lipoid density. Numerical estimations of lipoid density were not made, but sections of both glands from each mouse were compared repeatedly under the microscope. Gross differences in lipoid density were thus detected.

RESULTS.

The Ratio of Lipoid to Permanent Cortex (L/PC).

The values of L/PC and differences in lipoid density are shown in Fig. 1.

Explanation of Fig. 1.—Fig. 1 is composed of eight uniform diagrams. Ordinates denote permanent cortex. A broken line divides each diagram at the 0·5 level. Crosses denote left glands. Dots denote right glands in which lipoid density was the same as in the corresponding left glands. Circles denote right glands in which lipoid density was less than in the corresponding left glands. The two values for each mouse are in the same vertical line. When L/PC is the same in both glands of a pair the symbols are superimposed. The pairs are arranged from left to right in order of decreasing values of L/PC in left glands, and for each value of L/PC in left glands, in order of decreasing values of L/PC in right glands. Figures at the right of the diagrams indicate the interval in days between the removal of the left and right glands. N denotes normal mice from which both glands were removed simultaneously after death. C denotes controls whose right glands were examined 14 days after laparotomy.

Specimen reading—first pair at 14 days: Sudanophil lipoid occupied the entire width of the permanent cortex (L/PC = 1·0) in the left gland. In the right gland, removed 14 days later, it occupied 0·7 of the width of the permanent cortex (L/PC = 0·7). The lipoid was less dense in the right gland than in the left.

Criteria of lipoid change.—Since the amount of lipoid depends on (*a*) the area occupied by lipoid, of which L/PC is a measure, and (*b*) lipoid density, two criteria are applicable in deciding whether there is a significant difference between the relative amounts of lipoid in the left and right glands of a pair.

In a systematic study of the cortical lipoid in the mouse suprarenal (Whitehead, 1933), it was found that the difference between the values of L/PC in left and right glands of a pair was less than 0·3 in 95 per cent. of 59 pairs of glands from male mice of various colours and ages. In the remaining 5 per cent. the difference was 0·3. Determinations of L/PC in a fresh series of normal mice (Fig. 1, N) showed a maximal difference of 0·2 between the values of

L/PC in pairs of glands. These results show that the relative amounts of lipoid are usually about the same in both suprarenals simultaneously removed from one mouse. This fact explains why only one suprarenal was taken from each of the laparotomized mice (Fig. 1, C). The absolute amounts of lipoid depend, of course, on the amount of cortical tissue present in each of a pair of suprarenals.

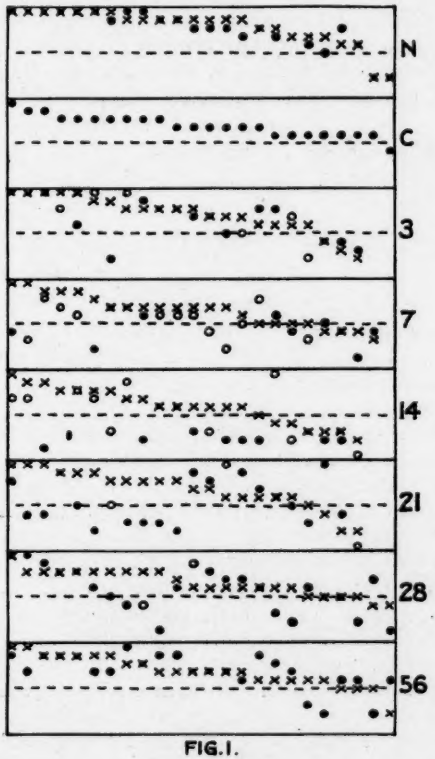


FIG. 1.

FIG. 1.—Ratios of lipoid to permanent cortex (L/PC). Explained in text. Ratios in right suprarenals below 0·5 (broken line) are rare in diagrams marked N, C and 56, frequent in remainder.

Since in the normal mice (Fig. 1, N) the difference between the values of L/PC in a pair of glands never exceeded 0·2, a difference of 0·3 or more in pairs of glands from unilaterally suprarenalectomized mice is considered to indicate a change in the amount of lipoid (criterion I).

Since in the normal mice lipoid density always appeared the same in both glands of a pair, any difference in density is considered to indicate a change in the amount of lipoid (criterion II).

Exception : Pairs in which the right gland showed decreased lipoid density, but had a value of L/PC greater by 0·3 or more than that for the corresponding left gland, are not considered to show a real difference in the amount of lipoid, since a large area with sparse lipoid droplets in the right gland is held to be roughly equivalent to a small area with closely packed lipoid droplets in the left.

Some pairs show a difference in both L/PC and lipoid density. These are reckoned as showing not two separate differences, but simply one difference in the amount of lipoid judged by two criteria instead of the usual one.

Table I, constructed from Fig. 1, according to these rules, shows the frequency of lipoid decrease after unilateral suprarenalectomy.

TABLE I.—*Percentages of Mice with Decreased Amount of Lipoid in Right Suprarenal at Various Intervals after Removal of Left.*

Intervals in days	3	7	14	21	28	56
Percentages of mice	36	61	68	45	33	17

Histological Types of Lipoid Change.

Decrease.—Three main types of lipoid decrease were seen.

Type I : Decrease in breadth of lipoid band without change in lipoid density (Figs. 2 and 3). The right gland shown in Fig. 3 would be denoted by a dot in Fig. 1.

Type II : Breadth of lipoid band unchanged, lipoid density uniformly decreased (Figs. 4 and 5). The right gland shown in Fig. 5 would be denoted by a circle in Fig. 1.

Type III : Decrease in breadth of lipoid band with decrease in lipoid density.

All three types were mentioned by Wolff (1927).

Increase.—Figs. 6 and 7 illustrate increase in both breadth of lipoid band and lipoid density.

As Figs. 3 and 7 show, changes in the breadth of the lipoid band are due to variations in the position of its inner border. In normal glands also, lipoid varies most in the inner part of the permanent cortex (Whitehead, 1933).

Relative Breadth of Cortex in Pairs of Glands.

The breadth of the permanent cortex (PC) was measured near each end of the minor axis in 29 pairs of suprarenals, the right gland of each pair being

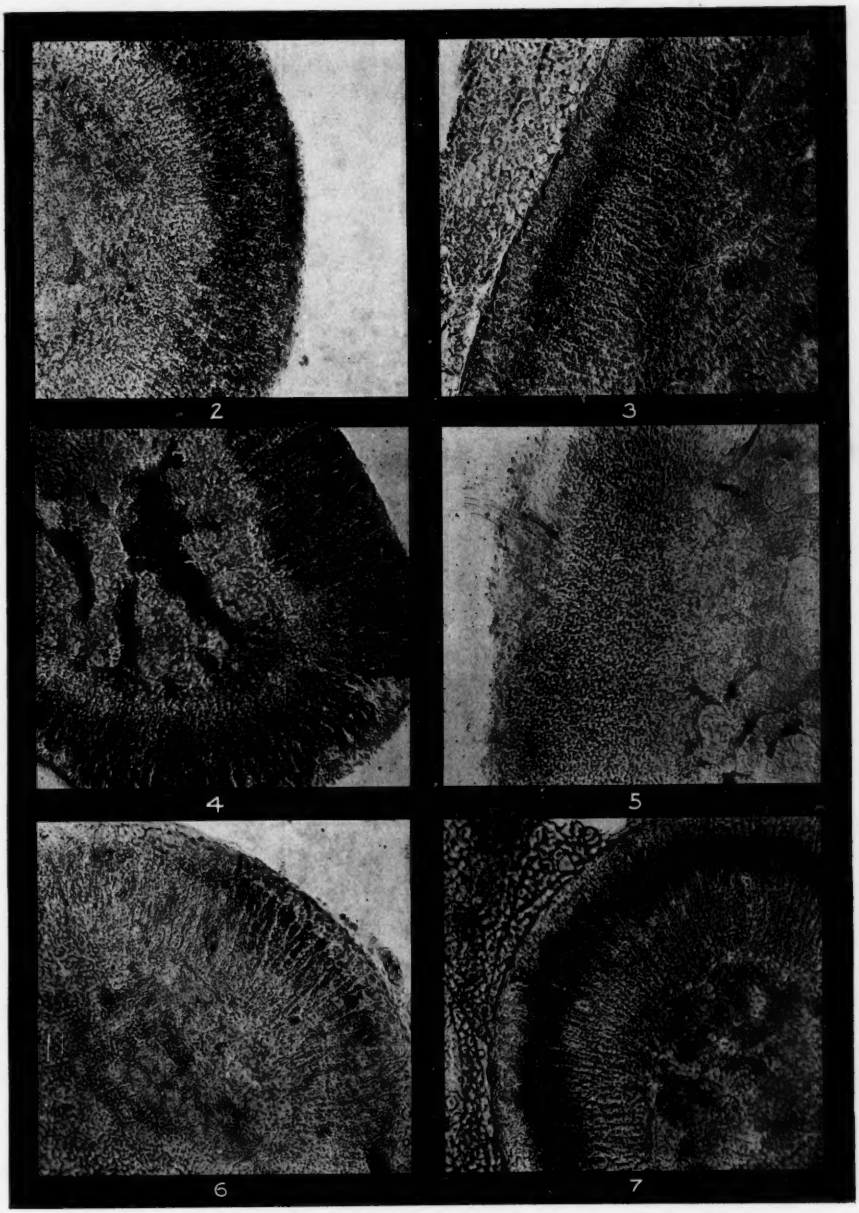
DESCRIPTION OF FIGURES.

FIGS. 2–7.—Sudanophil lipoid in frozen sections of pairs of suprarenals. Right glands removed from mice at the times stated after the left. Left glands (even numbers) on left of page. All × 80.

FIGS. 2, 3.—Lipoid decrease, Type I: Lipoid band narrowed, lipoid density unchanged. 14 days.

FIGS. 4, 5.—Lipoid decrease, Type II: Breadth of lipoid band unchanged, lipoid density uniformly decreased. 14 days.

FIGS. 6, 7.—Lipoid increase: Lipoid band broadened, lipoid density increased. 28 days. Photographs by H. C. Taylor.



Whitehead.

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removed 56 days after the left. The two readings on each gland were averaged. The results are shown in Table II.

TABLE II.—*Percentage Frequency of Various Differences between* PC *in Pairs of Suprarenals. Explained in Text.*

A	− 0·35 to − 0·11	− 0·1 to + 0·1	+ 0·11 to + 0·35	+ 0·36 to + 0·6
B	24	45	31	0
C	38	34	14	14

Explanation of Table II.—Table II contains three rows of figures. Row A denotes four ranges of differences of PC in right glands from PC in the corresponding left glands. Row B denotes the percentage frequency of right glands in each range. Row C denotes the percentage frequency of right glands in each range in a series of glands from 29 normal male mice aged 111 to 280 days.

Specimen reading—Row B, second entry: When PC in left gland is taken as 1·0, PC was between 0·9 and 1·1 in 45 per cent. of right glands.

Table II contains no evidence that hypertrophy of the permanent cortex had occurred in right glands removed 56 days after the left.

DISCUSSION.

The Nature of the Lipoid Changes.

The results show that Wolff (1927) underestimated the decrease in the amount of sudanophil lipoid occurring in the cortex of the suprarenal left *in situ* after unilateral suprarenalectomy. The proportion of right glands with decreased lipoid rose quickly to a maximum of 68 per cent. at 14 days after removal of the corresponding left glands, and fell gradually to 17 per cent. at 56 days. Since it is not known whether the amount of lipoid decreased at the same rate in different glands, it is not possible to say in what proportion of the total number of right glands lipoid decreased.

A few right glands removed from 21 to 56 days after the corresponding left glands show an increase in the amount of lipoid. These observations, and the fact that during the same period the proportion of right glands with a decreased amount of lipoid steadily fell, indicate that the decrease is temporary, and that the amount of lipoid in depleted right glands in time attains or exceeds its original value.

The Cause of the Lipoid Changes.

The series of values of L/PC in right glands removed 14 days after control laparotomy (Fig. 1, C) is indistinguishable from the series of values of L/PC in glands from normal mice (Fig. 1, N). Since 14 days after removal of the left glands was the epoch at which the maximal proportion of right glands showed a decreased amount of lipoid, it is demonstrated that the lipoid changes are actually due to removal of the left suprarenal, since every other procedure was common to both suprarenalectomies and laparotomies.

Lipoid Changes as Sources of Error.

Wolff (1927) illustrated suprarenals from mice that had received thyroxin, mouse typhus bacilli, olive oil, and lecithin, and also from mice starved for several days (his figs. 2–4). The left suprarenals were removed by operation. The right suprarenals, obtained at unstated intervals after the left, all show a decrease in the amount of lipoid. The possibility that this decrease may have been due to removal of the left gland cannot be excluded.

Deanesly (1931) described changes in the cortical lipoid in suprarenals from mice after injections of killed *B. Gaertner*, thyroxin and morphine. Her figs. 2, 6–10, 15, 16, 19 and 20 are alleged to illustrate experimentally induced lipoid changes. I have seen all the types of lipoid distribution there shown in suprarenals from normal mice (Whitehead, 1933). Her figs. 6 and 20 are alleged to show a reduction in lipoid, her figs. 7 and 10 an increase. All four figures illustrate types of lipoid distribution commonly found in normal glands, and in the absence of evidence as to the amount of lipoid present before giving the toxic agent, obviously no conclusion is possible. Her figs. 1 and 2, and 14 and 15, show pairs of glands, the right gland of each pair being removed from 7 to 11 days after the left. The differences between these pairs of glands are indistinguishable from those common at this interval after unilateral suprarenalectomy alone.

SUMMARY.

1. The relative amounts of sudanophil cortical lipoid in both suprarenals of 137 unilaterally suprarenalectomized male white mice have been estimated.

2. Right suprarenals removed at intervals from 3 to 56 days after the corresponding left glands showed a decrease in the amount of lipoid.

3. The proportion of right glands with decreased lipoid rose to a maximum of 68 per cent. at 14 days after removal of the left glands, and gradually fell to 17 per cent. at 56 days.

4. Some right glands showed increased lipoid from 21 to 56 days after removal of the left glands.

5. Control laparotomies on 24 mice demonstrate that the lipoid changes are not due to the surgical technique.

6. The results indicate that the amount of lipoid in depleted right glands in time attains or exceeds its original value.

7. Histological types of lipoid change are described and illustrated.

8. Lipoid changes as sources of error are discussed.

9. No evidence of cortical hypertrophy in 29 right glands removed 56 days after the left was obtained.

I am indebted to Dr. A. W. Downie for suggesting urethane-ether anæsthesia ; to Prof. S. L. Baker for advice and criticism ; and to the University Committee on Grants in Aid of Medical Research for defraying expenses.

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THE CALCIUM CONTENT OF THE CEREBRO-SPINAL FLUID IN NORMAL PREGNANCY AND IN ECLAMPSIA.

DAVID FYFE ANDERSON.

From the Royal Maternity and Women's Hospital, and the Biochemical Laboratory, Royal Infirmary, Glasgow.

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CALCIUM ions are believed to play an important part in allaying the irritability of the tissues in which they are contained. Meltzer and Auer (1908) found that calcium antagonizes abnormal activity in the body, be this abnormal activity exaggerated inhibition or exaggerated excitation. Following on the observation that a very large percentage of the cases of eclampsia already investigated showed a subnormal serum calcium value (Anderson, 1932), it was decided to estimate the calcium content of the cerebro-spinal fluid in the hope that variations in concentration might throw some light on the occurrence of convulsions.

Merritt and Bauer (1931) considered the cerebro-spinal fluid calcium content to be a more accurate physiological representation of the diffusible calcium of the serum than the values obtained by the various filtration methods used to determine diffusible calcium. McCance and Watchorn (1931), while mentioning that the cerebro-spinal fluid is generally considered to contain about 5 mgm. of calcium per 100 c.c., quote authorities to the effect that variations are normally from 4·4–6·6 mgm. per 100 c.c. They estimated the calcium in the cerebro-spinal fluid to be, on the average, 54·7 per cent. of that of the serum. These workers (Watchorn and McCance, 1932) state that opinion is still divided as to whether ultra-filtration will wholly explain the calcium in the cerebro-spinal fluid, but, they add, there is no doubt that, when a series is averaged, the cerebro-spinal fluid calcium and the ultra-filtrable calcium lie very close together, and that both tend to rise and fall with the total serum value. They only studied one case of eclampsia, and found it presented no abnormalities. Peters and Van Slyke (1931) state that the cerebro-spinal fluid contains about 5·0 mgm. of calcium per 100 c.c., and that the decrease in calcium of exudates and spinal fluid as compared with serum appears to parallel the protein content. These estimates are borne out by the investigations of Salvesen and Linder (1923), Gollwitzer-Meier (1925), and Schade and his co-workers (1926). Hunter (1931) places the cerebro-spinal fluid calcium level at 5–6 mgm. per 100 c.c., and states that it is evident that the calcium in the ultra-filtrates considered as a percentage of the serum calcium is about equal to the cerebro-spinal fluid calcium expressed on the same basis.

TABLE I.—*Calcium Content of Cerebro-spinal Fluid in Eclampsia* (16 *Cases*).

Case No.	Age.	Gravida.	Type of eclampsia.	Number of fits.	Mode of delivery.	Child. (M. = male. F. = female.)	C.S.F. calcium mgm. per 100 c.c.	Result to mother.
1	20	1	Post-partum	5	Spontaneous	M., alive, 6 lb.	4·1	Recovery.
2	37	2	Ante-partum	6	Instrumental	F., alive, 8 lb.	5·0	,,
3	40	6	,,	5	Induction, spontaneous	M., dead, 4½ lb.	5·1	,,
4	26	1	,,	5	Ditto	M., alive, 3 lb.	5·1	,,
5	21	3	,,	23	Instrumental	F., dead, 5 lb.	5·1	Death.
6	15	1	,,	4	Spontaneous	F., alive, 7 lb.	5·1	Recovery.
7	32	3	,,	7	Induction of abortion		5·1	,,
8	28	1	,,	5	Hydatidiform mole		5·2	,,
9	17	1	,,	5	Spontaneous	Anencephalic, spina bifida. M., 2 lb.	5·2	,,
10	26	1	,,	3	Instrumental	M., alive, 4½ lb.	5·3	Death.
11	39	5	Post-partum	4	Spontaneous	M., alive	5·5	Recovery.
12	34	4	,,	16	,,	Alive	5·5	,,
13	27	1	Intra-partum	4	Instrumental	F., alive, 7 lb.	5·5	Death.
14	27	1	Post-partum	3	Spontaneous	M., alive, 5½ lb.	5·6	Recovery.
15	26	1	Ante-partum	10	Instrumental	F., dead, 3 lb.	5·8	Death.
16	26	1	Intra-partum	14	,,	M., dead, 8 lb.	5·9	,,

Average value = 5·3 mgm. per 100 c.c.

METHOD AND SOURCE OF MATERIAL.

The method employed was that of Kramer and Tisdall (1921), as modified by Clark and Collip (1925).

The cerebro-spinal fluids were obtained by lumbar puncture performed in the routine investigation of the cases of eclampsia. Other specimens were secured in a similar manner for diagnostic purposes, or, as in the cases of physiologically uncomplicated pregnancy requiring intervention (Cæsarean section, forceps delivery, etc.), opportunity was taken to carry out the operative procedure under spinal anæsthesia.

RESULTS.

Table I shows the findings in 16 cases of eclampsia. The values obtained in normal pregnancy appear in Table II.

TABLE II.—*Calcium Content of Cerebro-spinal Fluid in Cases of Pregnancy Requiring Instrumental or Other Interference on Account of Disproportion, but otherwise Normal in All Respects* (27 *Cases*).

Number of cases.	Cerebro-spinal fluid calcium mgm. per 100 c.c.
1	4·3
2	4·7
1	4·8
3	4·9
7	5·0
4	5·1
2	5·2
1	5·4
1	5·5
1	5·6
2	5·7
1	6·1
1	6·2
Total 27	Average value = 5·2 mgm. per 100 c.c.

DISCUSSION.

In 16 cases of eclampsia the lowest recorded value for the cerebro-spinal fluid calcium was 4·1 mgm. per 100 c.c. in a case of the post-partum type with five fits, while the highest value was 5·9 mgm. per 100 c.c. in a case of the intra-partum type with fourteen fits, which terminated fatally. The average figure for the 16 cases was 5·3 mgm. per 100 c.c.

Incidentally, in this group of 16 unselected cases there were 5 maternal deaths (31 per cent.), while a live child was born in 9 instances (56 per cent.).

In a group of 12 cases taken at random, comprising various conditions, such as nephritic and pre-eclamptic toxæmia, twin pregnancy, and including a case of uræmia in a non-pregnant woman, the calcium of the cerebro-spinal fluid ranged from 4·6 to 5·3 mgm. per 100 c.c., giving an average of 5·1 mgm. per 100 c.c.

The cerebro-spinal fluid calcium in 27 cases of normal pregnancy ranged from 4·3 to 6·2 mgm. per 100 c.c., giving an average of 5·2 mgm. per 100 c.c.

These results in normal and toxæmic pregnancy fall within the physiological limits indicated. Watchorn and McCance (1932), who examined 12 cases of pregnancy, found the ultra-filtrable portion of calcium increased, and suggest that, during pregnancy, a larger amount of calcium is ultra-filtrable than in normal conditions. It is interesting to contrast the practically identical figures for cerebro-spinal fluid calcium in normal pregnancy and in eclampsia with the subnormal serum calcium in 17 per cent. of cases of normal pregnancy and in 82 per cent. of cases of eclampsia (Anderson, 1932). The constancy of the cerebro-spinal fluid calcium in association with variable diminutions in the calcium content of the serum is noteworthy as indicating that the diminution in the serum calcium level in toxæmic cases is due to a reduction in the non-diffusible calcium.

SUMMARY.

1. The cerebro-spinal fluid calcium content in 27 cases of normal pregnancy was found to be within physiological limits, the average being 5·2 mgm. per 100 c.c.

2. 5·1 mgm. per 100 c.c. was the average reading in a group of conditions, all except one associated with pregnancy (12 cases).

3. In 16 cases of eclampsia, while the maximal and minimal values were slightly lower than in the cases of normal pregnancy examined, physiological limits were not exceeded. The average was 5·3 mgm. per 100 c.c.

4. The original hypothesis was not substantiated. There was no abnormal variation in the level of the cerebro-spinal fluid in eclampsia which could be connected with the occurrence of convulsions.

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NOTE ON THE CEREBRO-SPINAL FLUID CALCIUM LEVEL IN INFANTS.

In the course of investigations on the cerebro-spinal fluid in normal and toxæmic pregnancy, the fluid obtained by lumbar puncture in infants a few days old, having convulsions, was examined with regard to its calcium content.

The tabulated results show the maximal and minimal values as 6·9 and 6·1 mgm. per 100 c.c. respectively, with an average of 6·5 mgm. per 100 c.c.

Calcium Content of Cerebro-spinal Fluid in Infants having Convulsions (6 Cases).

Case No.	Child.	Mode of delivery.	Age of mother.	Gravida.	Duration of labour.	C.F.S. calcium mgm. per 100 c.c.
1	Male, 6¾ lb.	Instrumental	28	1	61½ hours	6·1
2	Male, 6½ lb.	,,	24	1	16¾ ,,	6·2
3	Female, 3¾ lb.	Spontaneous	24	2	28 ,,	6·3
4	Male, 4½ lb.	Breech	34	3	3¼ ,,	6·5
5	Male, 6½ lb.	Instrumental	21	1	72¼ ,,	6·8
6	Female, 6¾ lb.	Spontaneous	31	2	13 ,,	6·9

Average value = 6·5 mgm. per 100 c.c.

Price (1926), referring to children, gives the calcium of circulating lymph, pleuritic, ascitic and spinal fluid as approximately 40–60% of the blood calcium. Levinson (1928), in connection with the cerebro-spinal fluid in infants and in children, mentions 4 mgm. per cent. as a low cerebro-spinal fluid calcium, 6·3 mgm. per cent. as a high cerebro-spinal fluid calcium, and the normal value as 5·2 mgm. per 100 c.c.

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THE BLOOD GROUP DISTRIBUTION IN THE EASTERN COUNTIES OF ENGLAND.

M. PENROSE AND L. S. PENROSE.

From the Research Department, Royal Eastern Counties' Institution, Colchester.

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THE systematic study of the distribution of the four blood groups in England has hitherto, in comparison with other countries, been somewhat neglected. Hirszfeld (1928) carried out an examination of 500 British soldiers, recruited from all parts of the country, including Wales, Ireland and Scotland. Since then a few other groups of figures bearing on this subject, for example, those of Buchanan and Higley (1921), have been recorded. Hirszfeld's subjects were, however, not a very homogeneous group as regards nationality, and so cannot be used as a standard for English people.

In the course of routine examinations of material for the study of the inheritance of mental characters, the present writers have grouped the blood of 1000 patients at the Royal Eastern Counties' Institution for Mental Defectives. These subjects were all drawn from homes in Essex, Suffolk, Norfolk or Cambridgeshire, and people of Jewish or foreign extraction were excluded.

The typing was carried out by a method almost the same as that of Hirszfeld. A few drops of the patient's blood were taken into 1 c.c. of normal saline containing 1 per cent. sodium citrate ; two drops of the suspension so obtained were then tested on a slide with drops of serum of group A and group B respectively. The serum supplied by Parke Davis & Co., from St. Mary's Hospital Inoculation Department, was used for the first few cases. Afterwards serum was obtained from two patients established as belonging to groups A and B respectively. Occasionally group A serum produced rather feeble agglutination. Group B serum always produced very definite results. In all doubtful cases the test was repeated.

In America the blood grouping of 300 feeble-minded patients was investigated by Snyder (1929), and the results (44 per cent. group O, 42 per cent. group A, 10 per cent. group B and 4 per cent. group AB) showed no appreciable deviation from the type of frequency shown in a systematic survey of 20,000 normal Americans (45 per cent. group O, 41 per cent. group A, 10 per cent. group B, 4 per cent. group AB). There is, therefore, no reason to suppose that feeble-mindedness makes any difference to the fairness of the sample. To make doubly sure of this, all mental defectives of the " mongolian " type were excluded.

Table I compares our results with certain other figures.

TABLE I.—*Distribution of Blood Groups in the Eastern Counties of England, Compared with the Distribution in Certain Other Localities.*

Population.	Number tested.	Percentage of persons in each group.				Percentage frequency of allelomorphs.			$p + q + r$.
		O.	A.	B.	AB.	p.	q.	r.	
Eastern counties of England (M. & L.S.P.)	1000	43·2	47·7	6·4	2·7	29·6	4·7	65·7	100·0
English soldiers (Hirszfeld, 1928)	500	46·4	43·4	7·2	3·0	26·8	5·2	68·1	100·1
Dutch students (Van Herwerden, 1930)	3085	45·7	41·2	9·6	3·5	25·6	6·8	67·6	100·0
Netherland State Prison (Van Herwerden, 1930)	691	43·7	45·4	8·7	2·2	27·6	5·6	66·1	99·3
Dutch farmers and fishermen (Penning, 1932)	389	44·2	45·5	6·2	4·1	29·0	5·3	66·5	100·8

The respective frequencies, p and q,* of the allelomorphic gene substitutions for the agglutinogens, A and B,* were obtained by the method of Bernstein as described by Lattes (1932) thus :

$$p = 1 - \sqrt{O + B},$$
$$q = 1 - \sqrt{O + A},$$

where O, A and B are the frequencies of these groups in the sample of the population studied. If $p + q + r = 1$, where $r = \sqrt{O}$, and $AB - 2pq = 0$, the sample is homogeneous. In our figures $p + q + r = 1{\cdot}000$, and $AB - 2pq = 0{\cdot}001$.

The frequency (p) of the allelomorph A to be found in the population of the Eastern Counties more nearly resembles the frequency found in certain parts of Holland than the frequency found for other parts of England. The frequency (q) of the allelomorph B in England is lower than that found in most other European countries. Low estimates have been also recorded in isolated districts in Holland. The figures for Dutch university students, many of whom were of foreign descent, do not, however, resemble the English Eastern Counties' frequencies so closely, although they do not differ greatly from Hirszfeld's English figures.

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* *A*, *B*, *p*, *q*, etc., here stand for frequencies, not percentage frequencies as in Table I.

IMMUNOLOGICAL STUDIES WITH THE VIRUS OF PSITTACOSIS.

S. P. BEDSON,
Senior Freedom Research Fellow.

From the Freedom Research Laboratory, London Hospital.

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In the course of a study of the virus of psittacosis the different aspects of immunity to this virus have been investigated. The findings form the subject of this paper.

SEROLOGICAL INVESTIGATIONS.

Neutralization.

Convalescent sera.—Early in this investigation the serum of human convalescents was examined for the presence of specific antibody by means of the neutralization test. None was found (Bedson and Western, 1930, *b*). This was in keeping with the observations of Rivers, Berry and Rhoads (1930), who concluded that the neutralizing power of convalescent sera was either absent or extremely feeble and subsequent work in this laboratory has confirmed this. However, the serum of mice which had been immunized with formalized virus and resisted a test with living virus was found to neutralize the virus (Bedson and Western, 1930), so an attempt was made to procure a potent serum by hyper-immunization. Since mice are obviously of no use for this purpose on account of their size, the guinea-pig was used.

Hyper-immune sera.—This animal is much less susceptible to psittacosis virus than the mouse. Intradermal inoculation produces a local reaction with, as a rule, no disturbance of general health. Even the intraperitoneal inoculation of 2 c.c. of a 5 per cent. suspension of virulent mouse spleen, containing many million of M.L.D. for the mouse, may elicit nothing more than a rise in temperature. This febrile response occurs, however, with regularity. Twenty-four hours after inoculation the temperature has risen to 105–106° F., falling to normal again by the third day after inoculation, and examination of the spleens and livers of these animals, both qualitatively and quantitatively, shows that the virus has multiplied, though not to the extent it does in the mouse. Passage in the guinea-pig did not increase the virulence of the virus for this animal species; by the third generation the virus had died out as a rule. Two guinea-pigs, P. 105 and P. 112, which had been used for the titration of virulent guinea-pig material, were hyper-immunized with suspensions of virulent guinea-pig's spleen. The dosage employed was 2–3 c.c. of a 10 per

cent. suspension given intraperitoneally at weekly intervals, with periodic rests of 1 to 2 months. These animals were bled from time to time to test their sera for antibody. They remained in excellent condition throughout. P. 105 was killed accidentally in performing cardiac puncture after it had received 10 inoculations; P. 112 has received 15. The neutralizing power of some of these samples of sera as tested in the guinea-pigs' skin and in the mouse is shown in Tables I and II.

TABLE I.—*Neutralizing Power of Hyper-immune Guinea-pig Sera Tested in Mice.*

Falling dilutions of a 5 per cent. virulent mouse spleen suspension mixed in equal quantities with (*a*) undiluted serum, (*b*) saline. After standing at room temperature for varying times a sample of each mixture was inoculated intraperitoneally in a mouse.

Serum.		Contact with virus.	Dose inoculated.	Final dilution of virus suspension.	Result in mouse receiving—	
Designation.	Number of immunizing doses.				(*a*) virus + serum.	(*b*) virus + saline.
P. 105	9	2 hours	0·5 c.c.	10^{-1}	D. 6	D. 4.
				10^{-2}	D. 10	D. 5.
				10^{-3}	D. 12	D. 6
				10^{-4}	D. 8	D. 7
,,	9	2 ,,	0·5 ,,	10^{-3}	D. 8	D. 7.
				10^{-4}	D. 12	D. 10
				10^{-5}	D. 15	D. 10
				10^{-6}	S. *nil*	D. 10
P. 112	12	3 ,,	0·2 ,,	10^{-4}	D. 16	D. 9
				10^{-5}	D. 13	D. 13
				10^{-6}	D. 12	S. v. ill
				10^{-7}	D. 14	S. v. ill
,,	13	2 ,,	0·5 ,,	10^{-2}	D. 7	D. 6
				10^{-3}	D. 7	D. 4*
				10^{-4}	D. 27	D. 10
				10^{-5}	D. 14*	D. 23
,,	13	24 ,,	0·5 ,,	10^{-2}	D. 8	..
				10^{-3}	D. 8	..
				10^{-4}	D. 34	..
				10^{-5}	S. *nil*	..
,,	14	2 ,,	0·5 ,,	10^{-2}	D. 10	D. 6
				10^{-3}	D. 10	D. 7
				10^{-4}	D. 10	D. 8
				10^{-5}	D. 19	D. 10

D. = Died and the number indicates the day after inoculation when death occurred.
S. = Survived.
* = Mixed infection.

Although the guinea-pig skin test shows that the sera of these animals have acquired definite neutralizing power, their efficacy in protecting mice is of an extremely low order. The different values given by these two methods of testing are probably due to the immensely greater susceptibility of the

TABLE II.—*Neutralizing Power of Hyper-immune Guinea-pig Sera Tested Intradermally in the Guinea-pig.*

Mixtures of virus and serum and saline made as in the mouse test (Table I). After 2 hours at room temperature 0·2 c.c. of each mixture was inoculated in the shaved hairy skin of a guinea-pig. Final reading taken on third day.

Serum.		Virus in serum. Final dilutions of virus suspension.				Virus in saline. Final dilutions of virus suspension.			
Designation.	Number of immunizing doses.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
P. 105	9	+	±	−	−	++	+	±	∓
P. 112	12	∓	−	−	−	+	±	∓	−
	13	±	−	−	−	+	+	±	∓
	14	±	−	−	−	++	+	±	?

The size of the lesion produced is indicated by the signs ++ to ∓.

mouse and the route of inoculation, the intraperitoneal route allowing dissociation of the virus-antibody complex which had not progressed further than loose union. This latter factor is suggested by a comparison of the results obtained in the mouse with serum P. 112 (bleeding after 13 inoculations) after 2 hours and 24 hours' contact with the virus before inoculation.

Complement-fixation and Agglutination.

Hyper-immune sera.—From what has been said it might be thought that the low neutralizing power of these hyper-immune guinea-pig sera was due to the samples tested being taken at the wrong time in the course of immunization. That this is not so is shown by the complement-fixation and agglutination reactions carried out with the sample bleedings. The agglutination reaction was made with washed elementary bodies following the technique already described (Bedson, 1932). The technique of the complement-fixation reaction was carried out according to the method of Bedson and Bland (1929). Specific antigen consisted of a 5 per cent. suspension of virulent mouse spleen, control antigens made from normal mouse spleen or the spleens of mice suffering from other virus infections (ectromelia) being invariably included in each test. Control sera from normal guinea-pigs or those immunized with herpes virus were also included. The results obtained were indubitably specific, and for the sake of brevity the controls are omitted from Table III, in which the complement-fixation findings are given. Table IV gives the results of agglutination.

Both showed a progressive rise in the antibody content of the sera of these two animals. Comparing these findings with the neutralization experiments, it is interesting to note that the serum of P. 112, although acquiring a considerable antibody content as evidenced by complement-fixation and agglutination, has little power of protecting the mouse, and that serum P. 105, poorer in antibody, protects the mouse as well or slightly better than P. 112. This is the converse of other antiviral sera worked with in this laboratory, in which neutralizing power has always appeared earlier and in greater

TABLE III.—*Showing Progressive Complement-Fixing Power in the Sera of Hyper-immunized Guinea-pigs.*

Guinea-pig.	Number of inoculations.	Dilution of serum. 1/1.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.
P. 105	6	++	−	−	−	..	..	..
		+	−	−	−	..	..	..
	8	+++	+	−	−	..	..	..
		++	±	−	−	..	..	..
	9	+++	+	−	−	..	..	..
		++	±	−	−	..	..	..
P. 112	6	+++	++	+	−	..	..	..
		++	+	−	−	..	..	..
	8	++++	+++	+	−	..	..	..
		+++	++	±	−	..	..	..
	9	++++	+++	++	+	..	..	..
		++++	++	+	−	..	..	..
	12	..	++++	++++	++++	++++	+++	..
		..	++++	++++	++++	+++	++	..
	14	..	++++	++++	++++	++++	+++	+
		..	++++	++++	++++	+++	+++	−

The signs ++++ to ± represent the degree of fixation. Readings taken after 30 minutes at 37° C. (upper row), and after standing overnight at room temperature (lower row).

TABLE IV.—*Showing the Progressive Development of Agglutinins in the Sera of Hyper-immunized Guinea-pigs.*

Guinea-pig.	Number of inoculations.	Dilutions of serum. 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
P. 105	6	++	+	+	−	−	..	..	..
	8	++	++	+	±	−	..	..	..
	9	+	+	++	+	−	..	..	..
P. 112	6	++	+	±	−	−	..	..	..
	8	+++	++	++	+	±	..	..	..
	9	+++	++	++	+	+	..	..	..
	14	++	++	+++	++	++	+	±	−

strength than complement-fixing power. Whether the divergence in complement-fixing and protecting power of the hyper-immune psittacosis sera is due to the virus containing more than one antigen, and the sera, in consequence, more than one antibody, is not known. There is evidence (Smith, 1932; Burnet, 1933) that some viruses possess an antigen resembling the soluble specific substance of bacteria. Possibly this may be so in the case of psittacosis virus.

Convalescent sera.—Earlier work has shown that convalescent sera may fix complement specifically despite the absence of detectable neutralizing antibody (Bedson and Western, 1930, *a*). In the light of the absence of parallelism between the neutralizing and complement-fixing power of hyper-immune sera the findings with convalescent sera become comprehensible. Further work on the complement-fixing power of convalescent sera, though limited to a few sera only, has confirmed the earlier observations.

In Table V the result of the titration of the complement-fixing power of two human convalescent sera is set out, and Table VI shows how this reaction may weaken as convalescence progresses.

TABLE V.—*Titration of Complement-Fixing Power of Two Human Convalescent Sera.*

Serum. Designation.	Dilution.	2 M.H.D. complement + 2·5 per cent. saline suspension of virulent mouse spleen diluted. 1/1	1/2	1/4	2 M.H.D. complement + saline.
"S"	1/1	++++	+++	+	−
		+++	++	±	−
	1/2	++++	+	−	..
		+++	±	−	..
	1/4	+	−	−	..
		±	−	−	..
	1/8	−	−	−	..
		−	−	−	..
"B"	1/1	++++	++	±	−
		+++	+	−	−
	1/2	++++	++	±	..
		++	+	∓	..
	1/4	++	±	−	..
		++	∓	−	..
	1/8	+	−	−	..
		±	−	−	..
Control 5	1/1	−	..	..	−
		−	..	..	−
„ 8	1/1	−	..	..	−
		−	..	..	−
„ 9	1/1	−	..	..	−
		−	..	..	−
Saline		−	..	..	−
		−	..	..	−

Sera inactivated at 56° C. for 20 minutes. Fixation for 3 hours at room temperature.

Another point brought out in Table VI is that sera from individuals who have not had psittacosis may give fixation. The control sera were obtained from amongst those sent in for the Wassermann reaction, and inquiry revealed the fact that the control serum 10 gave a positive Wassermann, whilst 8 and 11 were negative. On investigating this point it was found that a Wassermann positive serum quite frequently fixed complement in the presence of a psittacosis spleen suspension—and a normal spleen suspension also—whereas negative Wassermann sera did not do so. In using this test for diagnostic purpose one would have to do a Wassermann at the same time. The value of the

TABLE VI.—*Showing Weakening of the Complement-Fixing Power of a Serum in Convalescence.*

Serum. Designation.	Dilution.	2·5 per cent. saline suspension of virulent mouse spleen + 2 M.H.D. complement.	Saline + 2 M.H.D. complement.
"S"	1/1	++++	∓
		+++	−
	1/2	++++	..
		++	..
	1/4	+	..
		±	..
"B" 1	1/1	++++	−
		++++	−
	1/2	+++	..
		++	..
	1/4	++	..
		+	..
"B" 2	1/1	++	−
		+	−
	1/2	+	..
		∓	..
	1/4	−	..
		−	..
Control 8	1/1	−	−
		−	−
„ 10	1/1	++++	−
		++	−
„ 11	1/1	∓	−
		−	−
Saline		−	−
		−	−

Sera inactivated at 56° C. for 20 minutes. Fixation at room temperature for 3 hours. "B" 1 and "B" 2 were obtained from the same patient, the former 21 days and the latter 63 days after the onset of illness.

reaction as a diagnostic procedure is further detracted from by the fact that some sera from undoubted cases may fail to show fixation. Out of 6 sera examined by the writer 1 gave a negative result. Nevertheless this test, if carefully controlled, should prove of value in diagnosis, a positive result, as in most biological tests, being of much greater value than a negative one.

ACTIVE IMMUNIZATION.

Previous work (Bedson and Western, 1930, *b*) had suggested that virus rendered inactive by means of formalin was capable of evoking immunity. Although prophylactic immunization against psittacosis is of no practical

importance, work along this line has been continued because of the light which it might throw on immunization against filterable viruses in general. In both the earlier and subsequent experiments mice have been used, and any degree of protection achieved has all the greater significance because of the high susceptibility of the mouse to this virus. In Tables VII and VIII the details of two experiments are given.

TABLE VII.

Vaccine.—Suspension of virulent mouse spleen in saline. Titre in guinea-pig's skin 1 in 100. Divided into three portions:

A. Steamed for 20 minutes.
B. 0·2% formalin added and placed in refrigerator for 48 hours.
C. Treated like B and then steamed for 20 minutes.

Mouse.	Immunization.	Interval before test.	Test. 5% virulent spleen suspension M. 608/9.	Result.
576	Vaccine B: 3 doses of 0·3 c.c. i.p. at 7 days' interval	11 days	0·5 c.c. diluted 10^{-5} i.p.	D. 5*
577				D. 14
578				S. *nil*
579			0·5 c.c. diluted 10^{-6} i.p.	D. 11
580				S. *nil*
581				S. *nil*
582	Vaccine C: 3 doses of 0·3 c.c. i.p. at 7 days' interval		0·5 c.c. diluted 10^{-5} i.p.	D. 11
583				S. *nil*
584				S. *nil*
585			0·5 c.c. diluted 10^{-6} i.p.	D. 11
586				D. 18
587				S. *nil*
588	Vaccine A: 3 doses of 0·3 c.c. i.p. at 7 days' interval		0·5 c.c. diluted 10^{-5} i.p.	S. v. ill
589				S. v. ill
590				S. v. ill
591			0·5 c.c. diluted 10^{-6} i.p.	D. 16
592				D. 16
593				S. *nil*
614	None		0·5 c.c. 10^{-5} dilution i.p.	D. 7
615			,, 10^{-6} ,,	D. 8
616			,, 10^{-7} ,,	S. v. ill
617			,, 10^{-8} ,,	D. 11

* Died from bacterial infection; no virus found.

At first glance the results of the experiment in Table VII might suggest that the steamed vaccine rendered the mice as resistant as the formolized or the formalized and steamed one. It must be pointed out, however, that three of the survivors prepared with steamed virus became very ill when tested with active virus—in fact at one time they looked certain to die—whereas none of the survivors in the other two categories showed symptoms as the result of the test inoculation. The experiment tabulated in Table VIII was somewhat vitiated by the test virus proving rather a poor one, with the result that the smaller of the two test doses was insufficient to kill normal mice. However, confining one's attention to those mice receiving the larger test dose, it will be seen that none of the mice prepared with formolized or formolized and steamed virus succumbed to infection with the virus, whilst two out of three prepared with steamed virus died and the third became ill,

TABLE VIII.

Preparation of vaccine.—Suspension of virulent mouse spleen in phosphate. Titre in guinea-pig's skin 1 in 1000. Divided into three portions:

A. Steamed for 20 minutes.

B. 0·1% formalin added and placed in refrigerator for 9 days.

C. Treated like B and then steamed for 20 minutes.

Mouse.	Immunization.	Interval before test.	Test. 5% virulent spleen suspension M. 700.	Result.	Interval.	2nd test.	Result.
661				D. 26*			..
662	Vaccine B: 3 doses of 0·3 c.c. s.c. at 7 days' interval	22 days.	Diluted 10^{-4} 0·5 c.c. i.p.	S. *nil*	2 months after first test.	Survivors given 0·5 c.c. of a 5% suspension which killed normal mice in 3 days.	S. *nil*
663				S. *nil*			D. 9
664				D. 17			..
665			Diluted 10^{-5} 0·5 c.c. i.p.	S. *nil*			D. 6*
666				S. *nil*			S. *nil*
667				S. *nil*			D. 3
668	Vaccine C: 3 doses of 0·3 c.c. s.c. at 7 days' interval		Diluted 10^{-4} 0·5 c.c. i.p.	S. *nil*			D. 4
669				S. *nil*			D. 7
670				D. 17			..
671			Diluted 10^{-5} 0·5 c.c. i.p.	D. 26			..
672				D. 33			..
673				D. 14			..
674	Vaccine A: 3 doses of 0·3 c.c. s.c. at 7 days' interval		Diluted 10^{-4} 0·5 c.c. i.p.	D. 15			..
675				S. ill			D. 7
676				S. *nil*			D. 4
677			Diluted 10^{-5} 0·5 c.c. i.p.	S. *nil*			D. 4
678				S. *nil*			D. 5
707			Diluted 10^{-4} 0·5 c.c. i.p.	D. 11			..
708				D. 11			..
709			Diluted 10^{-5} 0·5 c.c. i.p.	S. *nil*			D. 3
710	None			D. 3*			..
711			0·5 c.c. 10^{-4}	D. 11			..
712			,, 10^{-5}	S. ill			D. 4
713			,, 10^{-6}	S. *nil*			D. 3
714			,, 10^{-7}	S. *nil*			D. 3

* Died from bacterial infection; no virus found.

but recovered. It is interesting to note, too, that whilst none of the mice prepared with steamed virus developed any symptoms on receiving the smaller test dose—behaving, in fact, like normal mice—deaths occurred in the other two categories of mice from this dose of virus. The second test carried out on the survivors points to the formolized vaccine as being the best, for only one out of the four survivors in this group died from this enormous dose of virus, whereas all the other survivors succumbed. Summing up the results obtained from the experiments with the different vaccines, and excluding all immunized mice which did not receive a test dose of virus certainly lethal for normal mice, one gets the following figures:

Vaccine.	Number tested.	Number surviving.	Number of survivors showing illness.
Formolized	20	14	0
Formolized and steamed	9	7	0
Steamed	9	5	4
Normal controls	11	0	0

When one considers the very high susceptibilty of the mouse for the strain of virus used in this work, it is evident that, despite the limited number of animals used, virus inactivated by small concentrations of formalin can evoke quite a high degree of immunity. It would appear, too, that formolized virus that has been steamed is still antigenically active, which is in keeping with similar experiments with herpes virus (Bedson, 1931). Owing to the very few mice which recover once they have been infected with this strain of virus, sufficient animals have not been available to enable a comparison to be made between the immunity produced by formolized vaccines and that resulting from the natural disease. From the few recovered mice that have been tested, the impression has been gained that the natural disease gives a more solid immunity.

SUMMARY.

1. The sera of individuals convalescent from psittacosis possess no demonstrable neutralizing power, but will fix complement in the presence of a psittacosis antigen.

2. Similarly the sera of hyper-immunized guinea-pigs contain considerable antibody demonstrable by complement-fixation or agglutination, but only a low neutralizing power.

3. The value of the complement-fixation reaction in the diagnosis of psittacosis is discussed.

4. Virus which has been rendered inactive by means of formalin can evoke a considerable degree of immunity in the mouse.

5. Formolized virus can be steamed for 20 minutes without much loss of immunizing power, whereas steaming alone has a deleterious action.

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HAY FEVER. I.—A STUDY OF REAGIN-ALLERGEN MIXTURES.

DAVID HARLEY.*

From the Inoculation Department, St. Mary's Hospital, London.

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In 1921 Prausnitz and Küstner showed that serum from a fish-sensitive person produced a local sensitization on being injected into normal skins, thus demonstrating the presence of specific sensitizing substance in the allergic serum. Similar passive transfer of most forms of human hypersensitiveness (hay-fever, horse asthma, etc.) was reported subsequently by de Besche (1923), Freeman (1924), Coca and Grove (1925), and others. These allergic sensitizing substances have been termed *reagins*; the allergic exciting agent is designated the *allergen*.

The object of the present investigation was to examine the properties of mixtures of hay-fever reagin (serum from patients sensitive to *Phleum pratense* pollen) with its specific allergen (*Phleum pratense* pollen extract).

Reagin-Allergen Mixtures Produce Reactions in Normal Skins.

1. *Reagin with excess of allergen.*—It was found that the injection of such mixtures into normal skins caused reactions with regularity. The reactions differed somewhat from the usual "triple response" to the injection of allergen into the sensitive skin. Mixtures of hay-fever serum with pollen extract and saline were prepared as in Table I. Fig. 1 represents the reactions produced by intradermal injection of 0·15 c.c. of mixtures A and B into the arm of a normal person. After an interval of 10 minutes the injection wheals had increased but slightly and showed a little surrounding erythema. Starting at 10–15 minutes from the time of injection and reaching its maximum at 25–30 minutes the wheal of mixture A extended; in the process of extending its edges became less definite, and its white colour became erythematous; a well-marked flare developed. At 50 minutes the reaction was beginning to fade. Meanwhile mixture B had become absorbed as indicated. Controls of normal serum with pollen extract, and saline with pollen extract, were negative (the latter are not shown in Fig. 1, but were similar to reaction B). Injection of reagin-allergen mixtures, using serum from cases with very marked skin sensitivity, produced reactions which were more extensive than the above, and commenced rather earlier. (These tests and others to be described were repeated at least three times.)

TABLE I.

	8 parts.		1 part.
A.	Hay-fever serum	+	Pollen extract (5000 units per c.c.).
B.	,, ,,	+	Saline.

The unit of pollen extract (Noon and Freeman) is the amount extracted from one-millionth (0·000001) of a gramme of pollen.

* Working with a whole-time grant from the Asthma Research Council.

The explanation of the difference between the reaction of mixture A and the more clean-cut wheal produced in 10–15 minutes by the action of pollen extract on the previously sensitized skin would appear to be that in the former the reaction was taking place by degrees, the cells which were sensitized first being acted upon by the allergen before the slightly more distal cells had entered into combination with the remaining reagin. Coca and Grove (1925), Levine and Coca (1926), Clarke and Gallagher (1926), and Baldwin (1930), stated that reagin-allergen mixtures gave no specific reactions in normal skins. Gay and Chant (1927) and Foran and Lichtenstein (1930) reported the

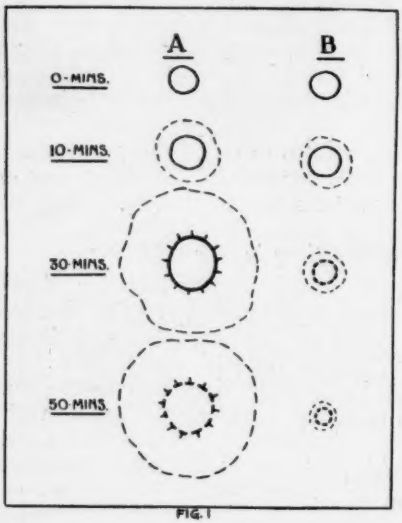

FIG. 1

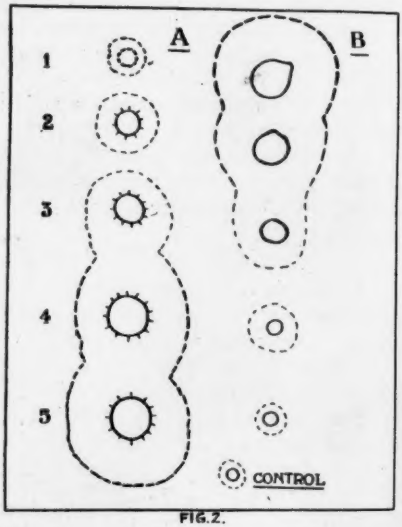

FIG. 2.

production of reactions with such mixtures. Indeed, Freeman finds that reagin-allergen mixtures react with such regularity that he has named them "urticarial mixtures".

2. *Reagin with various quantities of allergen.*—Mixtures of serum with pollen extract were prepared in the proportions shown in Table II. In the forearm of a normal person 0·1 c.c. of each mixture was injected intradermally, No. 1 most distally. Fig. 2, A, represents the reactions 30 minutes after injection when they were at their maximum. Six hours later, when the reactions had disappeared completely, 0·02 c.c. of pollen extract (2000 units per c.c.) was injected into each site through the original needle track, and into a normal unsensitized site as a control. In 10 minutes reactions developed as shown in Fig. 2, B.

The reaction produced by injecting the reagin mixed with allergen was of the same magnitude as that in which the reagin was injected first and followed

by the allergen in 6 hours (Fig. 2, A5 and B1). It shows further that only where there was an insufficient amount of allergen in the mixture to give the maximum reaction did reinjection of the site with allergen produce a second reaction. The quantity of allergen required to produce the maximum reaction with the serum used is seen to be about 40 units per c.c. of serum (the reinjection into site 4 gave a slight erythema only).

TABLE II.

Hay-fever serum 4 parts + pollen extract or saline 1 part.

Mixture.	Pollen extract (units per c.c. of mixture).	Reactions shown in Fig. 2 at site—
1	0·0 units	A1
2	0·4 ,,	A2
3	4·0 ,,	A3
4	40·0 ,,	A4
5	400·0 ,,	A5

Reagin is not Inactivated by Incubating at 37° C. with Excess of Allergen in vitro.

Three mixtures were made of hay-fever serum (4 parts) with 20,000 units per c.c. pollen extract (1 part). One mixture was incubated at 37° C. for 24 hours, one at 37° C. for 1 hour, the third was made up after the others had been incubated, and 0·1 c.c. of each was injected into skin sites of a normal person. A control injection of incubated serum with saline was made also. The 30 minutes' reactions which developed at the three sites were equal in size. The control was negative.

One concludes that the reagin had not been inactivated by incubating with the allergen. The neutralization of reagin by allergen would appear to take place only *in vivo*, that is, by a reaction involving tissue-cells. This conclusion is in agreement with that of Foran and Lichtenstein (1931), but is contrary to the view of Coca and Grove (1925), and Levine and Coca (1926). They observed the reactions caused by injecting allergen into skin sites which had received reagin-allergen mixtures previously. As they reported only the reactions following the reinjection of allergen (comparable to Fig. 2, B), and did not appear to obtain any reactions to the reagin-allergen mixtures themselves (comparable to Fig. 2, A), they came to the conclusion that reagin was neutralized by allergen *in vitro*.

Reagin is not Inactivated by Incubating with Excess of Allergen in the Presence of Blood-Cells.

Blood was obtained from a hay-fever patient, mixed with one-fifth of its volume of 5000 units per c.c. pollen extract (containing no carbolic or other preservative liable to damage the leucocytes), and incubated at 37° C. for 2½ hours. The mixture was then placed in the ice-chest for 18 hours and the

serum collected. One-tenth of a c.c. of the serum was injected into the skin of a normal person. The reaction was not smaller than that produced by a control injection of the same serum incubated with a similar quantity of pollen extract without blood-cells.

The reagin content of the serum was therefore undiminished by incubating in the presence of blood-cells ; that would indicate that no reaction had taken place between blood-cells, reagin and allergen. This is the result one would have expected *à priori*, since the cells which take part in an allergic reaction suffer some degree of damage in the process, and there is no evidence to indicate any damage to the leucocytes while a hay-fever patient is receiving large doses of pollen extract in the course of treatment. Experiments were made of the effect of pollen extract on the phagocytosis of staphylococci by the leucocytes of normal and hay-fever bloods, using the methods of Wright (1921) and Fleming (1931). Many observations were made with various concentrations

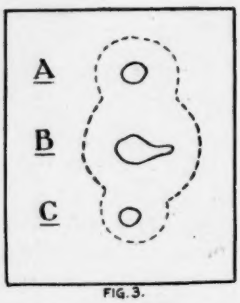

FIG. 3.

of pollen extract, but no difference was detected between normal and hay-fever leucocytes. Emigration of the leucocytes, as tested by the methods of Wright and Colebrook, was not affected by pollen extract to a greater extent than was that of normal leucocytes.

Allergen is Bound or Neutralized by Reagin in vitro.

Mixtures were prepared as shown in Table III. One-twentieth of a c.c. of each mixture was injected into the skin of the hay-fever case whose serum had been employed in making the mixtures. The reactions produced are represented in Fig. 3.

This experiment shows that incubating allergen with reagin renders the former inactive to the hay-fever skin. (The fate of the inactivated allergen will be discussed later.)

TABLE III.

9 parts.		1 part.	Reactions shown in Fig. 3 at site.—
Hay-fever serum	+	Pollen extract (400 units per c.c.)	A
Normal serum	+	,, ,, ,, ,,	B
,, ,,	+	Saline	C

The mixtures were incubated at 37° C. for 18 hours.

Time and Temperature Necessary for the Binding of Allergen by Reagin in vitro.

Mixtures were prepared as in Table IV. A hay-fever patient received 0·05 c.c. of each mixture intradermally. The reactions produced are represented in Fig. 4. The largest reactions were Nos. 1 and 5, caused by incubated normal serum with pollen extract, and hay-fever serum with pollen extract (injected as soon as prepared), respectively. Nos. 3 and 4, reagin-allergen mixtures at room temperature, gave reactions scarcely larger than the control of serum by itself. Nos. 6, 7 and 8, though much less than 1 and 5, were larger than 3 and 4; evidence will be brought forward to show that this was due to a non-specific action of heat on the serum. No. 2 gave a medium reaction as shown.

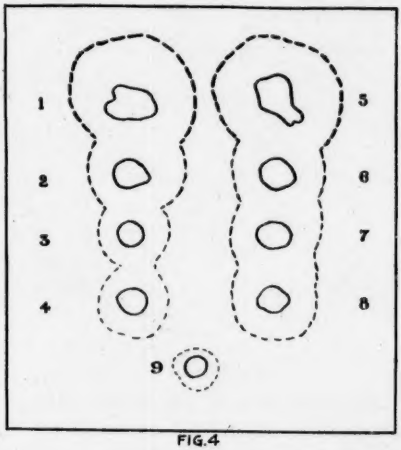

FIG. 4

The conclusion drawn was that the reagin had bound or inactivated the allergen at 37° C., at 18° C., and to a lesser extent at 4° C. This is contrary to the opinion of Levine and Coca (1926).

TABLE IV.

Serum 10 parts + pollen extract (500 units per c.c.) or saline 1 part.

				Reactions shown in Fig. 4 at site—
Normal serum	+	Pollen extract	: 24 hrs. at 37° C.	1
Hay-fever serum	+	,, ,,	: 24 ,, 4° C.	2
,, ,,	+	,, ,,	: 2 ,, 18° C.	3
,, ,,	+	,, ,,	: 24 ,, ,,	4
,, ,,	+	,, ,,	: Injected immediately	5
,, ,,	+	,, ,,	: 30 min. at 37° C.	6
,, ,,	+	,, ,,	: 2 hrs. ,,	7
,, ,,	+	,, ,,	: 24 ,, ,,	8
,, ,,	+	Saline	: 24 hrs. at 18° C.	9

Allergen Bound by Reagin can be Freed or Reactivated by Heating to Destroy the Reagin.

1. *The effect of heat on normal and hay-fever serum.*—Gay and Chant (1927) showed that normal serum produced reactions in normal and allergic skins after having been subjected to various degrees of heat. They found that 56° C. was the temperature which caused the serum to give the maximum reaction on injection. Less marked reactions were caused by heating to 60° C. and 38° C. They reported also that 56° C. caused a marked diminution in the reagin content of allergic serum.

TABLE V.

Serum 8 parts + pollen extract (2000 units per c.c.) or saline 2 parts.

1.	Hay-fever serum	+	saline	:	at room temperature.
2.	,, ,,	+	,,	:	at 60° C. for 1 hour.
3.	,, ,,	+	pollen extract	:	,, ,,
4.	,, ,,	+	,, ,,	:	at room temperature.

Reactions.

Mixture.	Time after injections.		
	10 mins.	30 mins.	1 hour.
1	—	—	—
2	+	faded	—
3	+	,,	—
4	—	++	+

Mixtures were made as shown in the upper part of Table V. The lower part of Table V indicates the reactions produced by the injection of 0·1 c.c. of each into a normal skin. Serum with pollen extract gave the usual reaction, maximal at 30 minutes. The same mixture heated to 60° C. for one hour produced a reaction in 10 minutes (due as stated to the action of heat on the serum) exactly similar to the heated control prepared with saline instead of pollen extract. The reactions 2 and 3 were not very marked, and had almost completely faded at 30 minutes.

This shows that the reagin had been destroyed by heating the serum to 60° C.

2. *To show that allergen bound by reagin can be freed by heating the mixture.*—Mixtures were prepared and treated as in Table VI. A hay-fever case received 0·05 c.c. of each mixture intradermally. The reactions produced are represented in Fig. 5. Comparison of reactions 2 and 6 show the distinctive effect of hay-fever serum on the allergen and the inappreciable effect of normal serum on the latter. Reaction 3 shows the effect of heating to 60° C. the mixture which produced the negligible reaction 2. This cannot be explained as due solely to the non-specific activity of heated serum referred to previously, since the control heated serum and saline gave only the slight reaction 4.

The conclusion drawn is that the allergen bound by reagin had been freed or reactivated to a certain extent by heating the mixture to 60° C.

TABLE VI.

10 parts.		1 part.				Reactions shown in Fig. 5 at site—
Hay-fever serum	+	saline	:	24 hrs. at 37° C.	. . .	1
,, ,,	+	,,	:	,, ,,	+ 1 hr. at 60° C. .	4
,, ,,	+	pollen extract	:	,, ,,	. . . .	2
,, ,,	+	,, ,,	:	,, ,,	+ 1 hr. at 60° C. .	3
Saline	+	,, ,,	:	,, ,,	. . . .	5
,,	+	,, ,,	:	,, ,,	+ 1 hr. at 60° C. .	8
Normal serum	+	,, ,,	:	,, ,,	. . . .	6
,, ,,	+	,, ,,	:	,, ,,	+ 1 hr. at 60° C. .	7
,, ,,	+	saline	:	,, ,,	. . . .	9
,, ,,	+	,,	:	24 hrs. at room temperature	. .	10

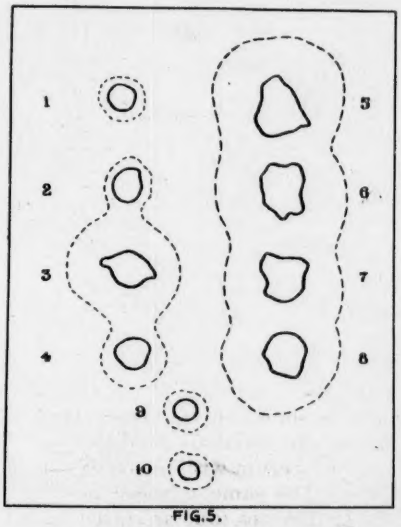

FIG. 5.

The Reactions of a Reagin-Allergen Mixture in Normal and Hay-fever Skins.

Mixtures were made of pollen extract with normal serum and with hay-fever serum, the pollen content being 40 units per c.c. of mixture. The mixtures were incubated to bind the allergen in the hay-fever serum mixture, and were divided into two portions. One portion of each mixture was heated to 60° C. for one hour. The four preparations were injected into a normal skin and into a hay-fever skin ; the reactions are indicated in Table VII. This shows in compact form the various reactions previously discussed. Hay-fever skin reacts to pollen extract in the normal serum mixture 1, but not to hay-fever serum mixture 2, in which the allergen has been bound. Normal skin gives the reverse. Heated hay-fever serum mixture does not react in the normal skin as the reagin has been destroyed, but reacts in the hay-fever skin, the allergen having been liberated by removal of the reagin.

TABLE VII.

		Hay-fever skin.	Normal skin.
Unheated	1. Normal serum + pollen extract	+	—
	2. Hay-fever serum + pollen extract	—	+
Heated	3. Normal serum + pollen extract	+	—
	4. Hay-fever serum + pollen extract	+	—

The reactions described in this paper are represented in diagrammatic form in Fig. 6.

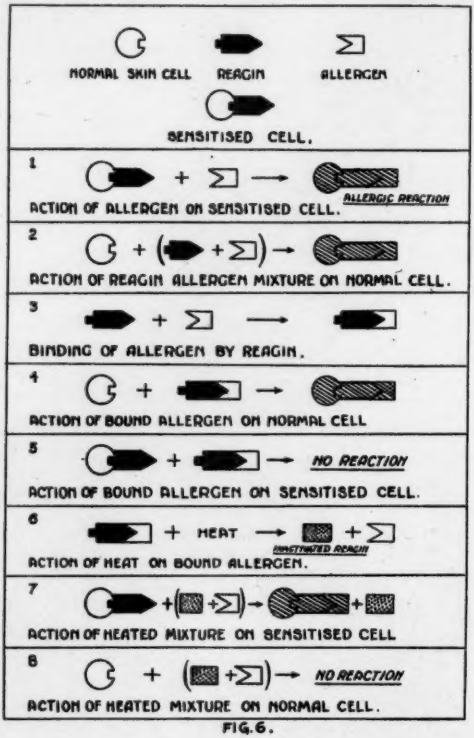

FIG. 6.

SUMMARY.

1. Reagin-allergen mixtures produce reactions in normal skins.
2. Reagin is not inactivated by allergen *in vitro*.
3. Allergen is bound by reagin *in vitro*.
4. Allergen bound by reagin can be freed by heating to destroy the reagin.

I wish to express my thanks to Dr. John Freeman for his kind assistance and to Mr. George Morgan for the preparation of the diagrams.

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THE FILTRATION OF RIFT VALLEY FEVER VIRUS THROUGH GRADED COLLODION MEMBRANES.

J. C. BROOM AND G. M. FINDLAY.

From The Wellcome Bureau of Scientific Research, London.

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A TECHNIQUE for the preparation of collodion membranes of regular pore size has been elaborated by Elford (1931, 1933), who, with his collaborators, has used them to determine the size of the particles of bacteriophage and of certain viruses.

In the following experiments we have used the methods described by him to estimate the probable size of the virus of Rift Valley fever, a disease of sheep and lambs first described by Daubney, Hudson and Garnham (1931). In confirming and extending their results, Findlay (1932) showed that the disease is almost invariably fatal in mice in from 2 to 4 days; mice were therefore used in our experiments to test for the presence of virus.

Material.

The heart-blood of mice dying of the disease was suspended in citrated saline or in nutrient broth and centrifugalized free of cells. In a few experiments an emulsion of liver in nutrient broth was used after centrifugation and filtration through sand and paper pulp (Barnard and Elford, 1931); in spite of this preliminary filtration the results obtained were less satisfactory, and its use was discontinued.

Although the virus retains its pathogenicity in 1 per cent. citrate saline for several weeks when kept at 4° C., inactivation occurred in certain experiments when it was washed repeatedly on a filter with citrated saline. Elford (personal communication) found a similar effect when a broth suspension of bacteriophage was washed with saline, and suggested that some stabilizing

substance might be lost during the washing. Inactivation did not occur when broth was used instead of citrated saline.

Elford noted in the case of dyes that the first fractions of filtrate contained minimal amounts of dye owing to its adsorption by the membrane, and that equilibrium had to be established before the filtrate contained the same concentration as the original suspension. This effect was well marked when a suspension of Rift Valley fever virus was filtered through membranes of pore size near its limit of permeability. When the filtrate was collected in small quantities for separate test it was found that the first fractions contained no virus, as shown by subcutaneous or intraperitoneal inoculation into mice; only after a considerable amount of fluid had passed through could the virus be demonstrated in the filtrate. For this reason the original suspension was not tested to titre in certain cases.

Table I gives the conditions of filtration and the results obtained in a series of experiments, from which it appears that the virus passes undiminished through a membrane of A.P.S. 0·15μ or larger, but is held back to some extent by one of A.P.S. 90μμ, and is completely retained by a pore size of 70μμ.

TABLE I.

Material.	Infectivity of stock suspension. (Dose 0·25 c.c.)	Membrane.	Pressure.	Volume filtered c.c. per sq. cm.	Test of filtrate. (Dose 0·25 c.c.)
Liver suspension	10^{-4} (2–4 days)	0·45μ	40 cm. Hg.	4	10^{-2} (2–4 days)
Ditto	,,	0·45μ	20 ,,	4	10^{-2} (3–5 days)
Blood suspension	10^{-4} (2–5 days)	0·35μ	40 ,,	4	10^{-4} (2–4 days)
Ditto	,,	0·35μ	20 ,,	4	10^{-4} (4 days)
,,	,,	0·35μ	40 cm. H_2O	4	10^{-2} (2–3 days)
,,	,,	0·25μ	40 cm. Hg.	4	10^{-3} (4 days)
,,	,,	0·15μ	40 ,,	4	10^{-4} (4 days)
,,	,,	0·15μ	20 ,,	4	10^{-3} (3 days)
,,	10^{-4} (2 days)	0·15μ	40 ,,	4	10^{-4} (2–4 days)
,,	10^{-6} (2–3 days)	90μμ	40 ,,	8	10^{-2} (3–4 days)
,,	10^{-6} (4 days)	90μμ	40 ,,	8	10^{-3} (4–5 days)
,,	Undiluted (2–3 days)*	80μμ	40 ,,	20	Undiluted (2–4 days)*
,,	Ditto*	80μμ	1 atmo.	20	Undiluted (2–3 days)*
,,	,, *	80μμ	1 ,,	20	Undiluted (3–4 days)*
,,	10^{-4} (2 days)	70μμ	40 cm. Hg.	20	Undiluted 0.
,,	Undiluted (2–3 days)*	70μμ	1 atmo.	20	,,
,,	10^{-3} (2–5 days)	70μμ	2 ,,	20	,,

* Not tested to titre.

Elford's calculations show that this grade of membrane retains particles of 0·33–0·5, the average pore size; the diameter of the virus particles should therefore lie between 23 and 35μμ.

Concentration Experiments.

A few experiments were performed in an attempt to obtain either relative or absolute concentration of the virus above a membrane through which it could not pass.

10 c.c. of a 1 : 10 dilution of the original blood suspension in nutrient broth was filtered through a membrane of A.P.S. 70$\mu\mu$ until 9 c.c. of filtrate was collected ; a further 9 c.c. of broth was added to the 1 c.c. of suspension remaining above the membrane, and filtration continued. This was repeated four times in all, giving a final relative concentration of 10^{-5} of the original suspension.

The final 1 c.c. of suspension left above the membrane and each 9 c.c. of filtrate were tested for virus, and the membrane was ground up with broth for test also. It was found that the membrane had absorbed so much of the virus that it could not be demonstrated even in the undiluted supernatant fluid.

To overcome this loss by adsorption, 20 c.c. of the virus suspension was used, and after it had been reduced to 1 c.c. by filtration the additions of broth were made as before.

Table II shows the results of two such experiments in which 70$\mu\mu$ and 50$\mu\mu$ membranes were used.

TABLE II.

Material.	Infectivity of stock suspension.	Membrane.	Pressure.	Test of filtrate.	Test of supernatant fluid after 5 times dilution.	Relative concentration.
Blood suspension	10^{-6} (2–4 days)	70$\mu\mu$	40 cm. Hg.	Undil. 0	10^{-7}	$\times$ 10
Ditto	10^{-3} (2–4 days)	50$\mu\mu$	2 atmo.	,,	10^{-9}	$\times 10^{6}$

In one experiment, 50 c.c. of virus suspension was filtered through a 50$\mu\mu$ membrane. Filtration had practically ceased when about 47 c.c. had passed, so the remaining 3 c.c. was tested for virus content. The original suspension killed in a concentration of 10^{-4} in 2 to 5 days, while the fluid above the membrane killed at 10^{-5} in 2 to 4 days, so that some concentration probably had been obtained.

SUMMARY AND CONCLUSIONS.

1. " Gradocol " membranes described by Elford have been used to estimate the size of the virus of Rift Valley fever.

2. This appears to lie between 23 and 35$\mu\mu$.

3. Concentration of the virus has been obtained above membranes impermeable to it.

4. It is of interest that herpes virus, which was found by Elford, Perdrau and Smith (1933) to be between 0°1 and 0·15μ in size, and which should therefore theoretically come within the limits of ultra-violet light photography, produces intranuclear inclusions very similar histologically to those observed in Rift Valley fever, where the infective agent is apparently very much smaller.

We should like to record our gratitude to Dr. Elford for much valuable advice, and for demonstrations of his technique of preparing these membranes.

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ON SO-CALLED REVERSED SELECTIVE BACTERIOSTASIS.

LAWRENCE P. GARROD.

From the Department of Pathology, St. Bartholomew's Hospital, London.

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THE experiments to be described originated in a study* of the resistance of the *Streptococcus pyogenes* to various bacteriostatic agents, including dyes, the aim of which was to devise a medium for growing this organism selectively. Since the original work of Drigalski and Conradi (1902), who first used crystal violet for the purpose of inhibiting "cocci and other saprophytic bacteria", and of Churchman (1912, 1913), who studied the inhibitory action of gentian violet and other violet dyes on 138 varieties of bacteria, it has been recognized that dyes of this type have a markedly selective bacteriostatic action, and Churchman's work made it clear that susceptibility to this action was a property of Gram-positive bacteria. There was no suggestion that any dyes had an opposite selective action until Churchman's (1923, *a*, *b*) observations on the action of acid fuchsin. A culture from the heart's blood of a mouse dead of anthrax being contaminated with a Gram-negative bacillus, he treated an emulsion of the mixed culture with this dye, and obtained thence a pure culture of *B. anthracis*. In further experiments mixed suspensions of *B. anthracis* and *B. pyocyaneus* were treated with acid fuchsin at a temperature of 45° C., and with gentian violet; cultivation after the former treatment resulted in a pure culture of *B. anthracis*, after the latter in a pure culture of *B. pyocyaneus*. This result led Churchman to define the action of acid fuchsin on Gram-negative bacteria so exhibited as "reversed selective bacteriostasis". He thought it peculiar to a certain class of chemical substances, since he found that the same effect was produced by acriflavine and by sulfanilic acid, chromotropic acid, sodium ethylsulphonate, sodium phenolsulphonate, and ethylsulphonic acid, and since all these compounds have an SO_3 group in their molecule, he asserted (Churchman, 1922) that the possession of this group determined the reversed type of selective action.

The only Gram-positive organism used by Churchman in these experiments was *B. anthracis*, and in defining the phenomenon of reversed bacteriostasis he restricts it to mixtures of Gram-negative bacilli with "Gram-positive spore-bearers" or "Gram-positive sporing aërobes". He fails to state, however, whether the preparations of *B. anthracis* used in his experiments contained spores, and neither the medium used for the culture providing them nor the age of the culture itself are stated. Since the preparation was an "aqueous suspension" it was presumably obtained from a solid medium, and therefore probably contained spores. It is clear that if the survival of anthrax bacilli treated with acid fuchsin and other substances depends on the presence of spores, any comparison with the effect of these substances on Gram-negative

* Assisted by the Medical Research Council.

bacilli or any other non-sporing organism is pointless, except as illustrating the resistance of spores. There would be no good reason for relating susceptibility to acid fuchsin to the Gram reaction, or for supposing that there is anything specific in the action of acid fuchsin and other compounds containing an SO_3 group, since it might be expected that almost any germicide in a suitable concentration would produce the same effect, just as it could certainly be produced simply by the action of heat.

The possible part played by spores in Churchman's experiments was considered by Burke and Skinner (1924), who showed that treatment with acid fuchsin destroyed vegetative cells but not spores of *B. subtilis*, and conclude that " the reverse selective action attributed to acid fuchsin is to be explained on a basis of spore resistance ". In a later paper (1925), in which they also question the destruction of spores by gentian violet, they arrive at the more general conclusion that " there is ground for the belief that any dye destroying Gram-negative organisms will also destroy Gram-positive organisms, exclusive of spores ". Burke and Skinner used *B. subtilis* in place of *B. anthracis* in their experiments with acid fuchsin, and they failed to obtain a preparation free from spores (only agar slope cultures were used). To this extent their refutation of Churchman's conclusions is incomplete. In any case it seems to have escaped recognition as such, since Churchman in later publications (1926, 1928), although referring to Burke and Skinner's work, repeats his description of reversed selective bacteriostasis in the original terms, implying that it is a specific action depending on the Gram-reaction of the bacteria concerned, and exerted only by acid fuchsin and certain other substances. His observations are also quoted, and his interpretation of them accepted in recent standard works on bacteriology. It therefore appeared desirable to repeat Churchman's own experiments under conditions which would show whether his interpretation of them is justified.

TECHNIQUE.

Two procedures are described by Churchman for exerting the selective action of acid fuchsin: the admixture of equal volumes of a bacterial suspension and 1 per cent. aqueous acid fuchsin (Grübler), and the addition of 5 drops of a saturated solution of the dye to 20 drops of bacterial suspension. I have determined the solubility of acid fuchsin (Grübler) in water, and found it to be 45·8 gm. in 100 c.c.; it follows that the concentration of acid fuchsin in the mixture prepared by one of Churchman's methods is more than eighteen times greater than in the case of the other. The method in the following experiments was admixture with an equal volume of 1 per cent. dye, the mixture being then maintained at a temperature of 45° C. as described by Churchman. The preparation of bacteria used was a dilution in sterile distilled water of a broth culture, in which the actual numbers of living bacteria were determined by inoculating shake agar plate cultures, in which colonies were subsequently counted, with 1 c.c. each from a series of decimal dilutions. Similar cultures were made from dilutions of the mixture during the progress of the experiment, and from these the proportion of bacteria surviving at each stage could be calculated. The dilution of culture used for admixture with dye solution (usually 1 in 100) was so chosen that countable plates could be obtained from

the second, third or fourth dilution of it; thus the making of an excessive number of dilutions and cultures was avoided, but the dye was so diluted in the cultures as to avoid any possibility of inhibitory action.

Figs. 1 and 2 represent the results of exposing *B. coli* with *Staphylococcus aureus* to the action of 0·5 per cent. acid fuchsin (equal volumes of 1 per cent. dye and bacterial suspension) at 45° C. for two hours, and those of exposing *B. coli* and *Streptococcus pyogenes* to the action of 0·04 per cent. acriflavine at 37° C. for two hours. It is clear that the Gram-positive organism is in each case destroyed more rapidly than the Gram-negative, and any likelihood that

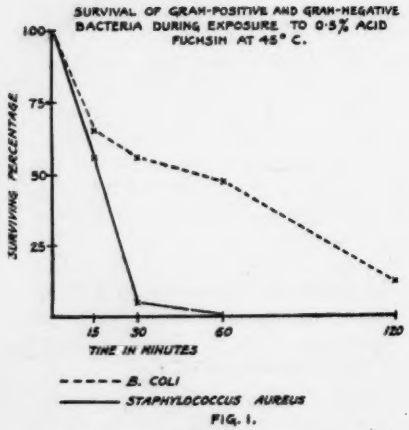

FIG. 1.

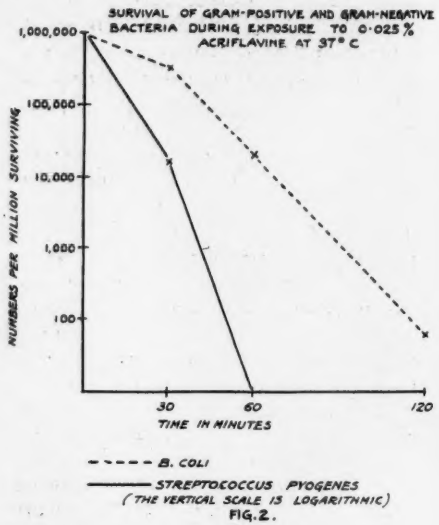

FIG. 2.

the selective action of acid fuchsin and acriflavine described by Churchman might have a general application in accordance with the Gram-reaction was dispelled by these results.

THE ACTION OF ACID FUCHSIN ON *B. ANTHRACIS*.

It remained to perform similar experiments with *B. anthracis*, and in particular to determine whether the survival of this organism depends on the presence of spores. It was found that if *B. anthracis* were maintained in broth, and subcultivated daily, a 20-hour culture was entirely free from spores (as indicated by complete sterilization at 80° C.). A dilution of such a culture in distilled water containing an adequate number of living bacilli (*e. g.* 130,000 per c.c.) was found to be completely sterilized by admixture with an equal volume of 1 per cent. acid fuchsin and exposure to a temperature of 45° C. within the short period of 15 minutes. In several repetitions of this experiment, not a single colony was ever obtained from any dilution of the mixture with acid fuchsin, even in the 15-minute cultures. It is therefore clear (*cf.*

Fig. 1) that vegetative anthrax bacilli are decidedly more susceptible than *B. coli* to the bactericidal action of acid fuchsin at this temperature, and the selective action of this dye is of the same nature as that of gentian violet, and not its reverse.

That survival of anthrax bacilli during exposure to acid fuchsin depends on the presence of spores was shown by using a 10-day broth culture for the same experiment. The number of surviving organisms falls abruptly at 15 minutes, and remains at approximately the same level throughout the period of exposure. It was expected that this number would correspond to the number surviving exposure to a temperature of 80° C. for 10 minutes (in the absence of acid fuchsin). This proved not to be so, since the number surviving treatment with acid fuchsin was always greater, *e. g.* of a suspension originally containing 490,000 living bacteria per c.c., 6·5 per cent. survived acid fuchsin for 2 hours, but only 0·3 per cent. survived heating to 80° C. The explanation for this discrepancy is evidently that spore-formation is a gradual process, and the full resistance of the spore is only developed slowly. A film of the culture used in this experiment stained by Moeller's method showed spores of which some were deep red, some a pale red, others almost colourless. It may be assumed that immature spores are resistant to acid fuchsin, but not to a temperature of 80° C. That this is so is suggested by the following experiment: A 20-day broth culture was heated to 80° C. to destroy vegetative bacilli and immature spores, and mixed in equal volumes with a 20-hour culture containing only vegetative bacilli. The numbers of both vegetative bacilli and heat-resistant spores were determined by decimal dilution and plating before and after heating a portion of the mixture to 80° C. A further portion of the mixture was treated with acid fuchsin; the result of this part of the experiment is represented in Fig. 3, where it will be seen that the number of surviving bacilli, after the initial fall in 15 minutes, remains approximately constant. The proportion of survivors here is 32 per cent.; the proportion surviving 80° C. was 16·2 per cent.—a difference much less than that observed with a natural 10-day culture; that it is still outside the limits of experimental error is perhaps attributable to the damaging effect even on comparatively mature spores of heating to 80° C. twice.

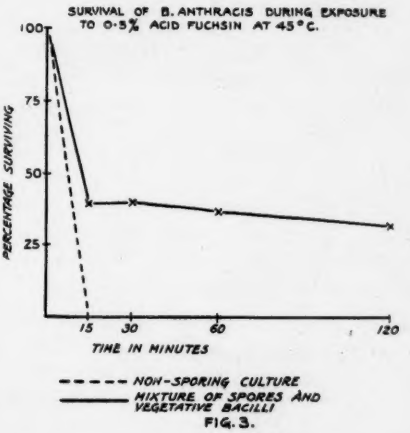

FIG. 3.

THE EFFECT OF A TEMPERATURE OF 45° ON *B. ANTHRACIS* AND OTHER BACTERIA.

The reasons for Churchman's choice of 45° C. as the temperature for treating suspensions of bacteria with acid fuchsin are not evident from his writings.

This temperature is so unusual that its action, apart from that of the dye, demanded investigation. A dilution of a 20-hour broth culture of *B. anthracis* in distilled water containing 6800 living organisms per c.c. was placed in a water-bath at 45° C. and cultivated after 2 hours, when it was found to have been completely sterilized. Since this unexpected result might have been due to the presence of a trace of copper in the distilled water, the experiment was repeated with dilutions made in glass-distilled water and in broth. In each case the same result was obtained. Finally decimal dilutions in broth were made from 20-hour cultures of each of four different strains of *B. anthracis*: the third, fourth and fifth dilution from each were placed at 45° C., and shake cultures from 1 c.c. of each dilution were made before exposure to this temperature and at intervals afterwards. The colony counts obtained are indicated in Table I.

TABLE I.—*Numbers per c.c. of* B. anthracis (4 *strains*) *surviving in Broth Dilutions kept at* 45° *C.*

	Strain.											
	1. Dilution.			2. Dilution.			3. Dilution.			4. Dilution.		
	3.	4.	5.	3.	4.	5.	3.	4.	5.	3.	4.	5.
Before heating .	+++	720	68	+++	++	194	+++	++	232	+++	++	310
After 30 mins. at 45° C. .	+++	219	12	+++	666	52	+++	++	86	+++	++	163
After 60 mins. at 45° C. .	0	0	0	283	12	0	0	0	0	++	8	0
After 120 mins. at 45° C. .	0	0	0	0	0	0	0	0	0	0	0	0

+++ = Many thousands. ++ = Over one thousand.

The following are particulars of the strains of *B. anthracis* used:

"Strain 1". Isolated by the late Sir Frederick Andrewes in this laboratory in 1905, and maintained here since. This strain was used for all the foregoing experiments.
"Strain 2". "Paddington VI". No. 109 in National Collection of Type Cultures; isolated 1919.
"Strain 3". "Hankow Hide". No 2620 in National Collection of Type Cultures; isolated 1928.
"Strain 4". "Prideaux". Isolated from a case of human anthrax at this Hospital in 1932.

It is clear from these results that vegetative anthrax bacilli, when exposed to a temperature of 45° C., even in a favourable medium, are destroyed. Complete sterilization is effected within one hour of all suspensions containing less than about 1000 bacilli per c.c.; in two of the four strains tested a proportion of the bacilli survived for one hour in stronger suspensions, but all were destroyed within 2 hours.

Pasteur (1878, 1881) cultivated anthrax bacilli at a temperature approaching this in order to produce his "premier vaccin". His findings were that the upper limit for growth was "below 44° C.", and that at "42–43° C." growth took place, but spores were not formed, virulence was to a large extent lost, and the cultures died out completely in about one month. In order to determine whether these growth conditions applied also to the four strains used in the tests described, an incubator was fitted with a capsule giving a range of

temperature from 40° to 45° C., and growth was studied at different temperatures within this range. Recognizable growth appeared at slightly higher temperatures on agar than in broth. Slight differences between the strains corresponded to the length of time which had elapsed since their isolation, the strain isolated as long ago as 1905 being the least tolerant of high temperatures. This strain failed to grow in broth, but grew on agar, at 43° C.; the remainder grew in both media at 43°; none grew at 44° C. The findings of Pasteur are thus confirmed for these four strains, and it is shown that a difference of only 2° C. separates the maximum temperature at which this organism will grow and the minimum temperature at which it is destroyed. That this difference should be so small is at first sight surprising, but it is possible that if other bacteria were studied in this way a similar result would be obtained. Many bacteria will remain quiescent for long periods at low temperatures; their period of survival is shortened at the optimum temperature for growth; above that temperature quiescence is inconceivable—they must either multiply or die. If this be granted, the range of temperature separating these effects will be large or small only in proportion to variations in resistance to heat possessed by individual organisms in a culture. The effect of exposure to 45° C. on some other bacteria was also studied, the dilutions of broth cultures used being made in both distilled water and in broth. The numbers of *Staphylococcus aureus* underwent a slight diminution only in 2 hours in both media. *B. coli* showed a slight diminution in distilled water, but multiplied in broth. *B. pyocyaneus* diminished slightly in broth and was destroyed in distilled water, the numbers found per c.c. being 880 before heating, 150 after 30 minutes, 19 after 60 minutes, and none after 2 hours.

DISCUSSION.

The experiments described show that vegetative anthrax bacilli are more susceptible to the bactericidal action of acid fuchsin under the conditions of Churchman's experiments than *B. coli*; they also show that vegetative anthrax bacilli can be destroyed merely by exposure to the temperature employed, in the absence of any dye. Anthrax spores, however, resist exposure to acid fuchsin under these conditions, and the conclusion is irresistible that the suspensions used by Churchman contained spores. His experiments, therefore, are simply an illustration of spore resistance, as much in this case to heat as to acid fuchsin, since *B. pyocyaneus* (one of the Gram-negative bacilli used by Churchman), as well as vegetative *B. anthracis*, can be destroyed simply by exposure to the temperature used. There is therefore no ground for crediting acid fuchsin with a "reversed selective" action, and other experiments have shown that both this dye and acriflavine share the same selective action as the violet dyes, although the difference between their effects on Gram-positive and Gram-negative bacteria is not so extreme.

There appears to be no authentic report of a reversal of this action by any dye. It seems that aniline dyes generally, in as far as their action on different bacteria varies, destroy Gram-positive more readily than Gram-negative. Although this principle is not generally recognized, it is implied in the

nomenclature proposed by Eisenberg (1918–19), who studied the bacteriostatic action of 240 salts on 6 Gram-positive and 6 Gram-negative bacteria. The majority of these salts inhibited the growth of Gram-positive bacteria in concentrations permitting that of Gram-negative, and this he describes as the " dye-stuff type " of action. Some, however, reversed this effect, among them being potassium tellurite (the use of which for inhibiting Gram-negative bacteria is a routine practice), and certain chromates, iodides, benzoates, etc. This effect Eisenberg calls the " inverse type " of bacteriostasis. This term claims recognition in preference to Churchman's " reversed bacteriostasis " both by priority and because it represents a reality.

Two other new terms have been suggested by Churchman (1923,*b*) : they are " extrinsic " and " intrinsic " bacteriostasis ; the former is illustrated by incorporating acid fuchsin in a culture medium, which then inhibits the growth of *B. anthracis*, but not that of *B. pyocyaneus*, the latter consists in treating the organisms with the dye in solution, and subsequently inoculating normal media, the result of which is a growth of *B. anthracis* but not of *B. pyocyaneus*. These experiments cannot be repeated, since they are insufficiently described, but without practical repetition their results can be explained by again assuming that the preparation of anthrax bacilli used contained spores. The resistance of these spores explains survival during exposure to the dye in solution ; the marked susceptibility of the vegetative bacilli to the action of the dye accounts for the failure of growth on media containing it. There is therefore nothing noteworthy or contradictory in these results, and no ground for distinguishing in nature between " extrinsic " bacteriostasis and " intrinsic ". The terms are bad both for this reason, and because " intrinsic " bacteriostasis is not bacteriostasis in the accepted sense of growth inhibition, but bactericidal action. The statement, "A substance, therefore, which ' kills ' an organism, when added directly to it, may have little or no power to inhibit its growth when present in the environment in which it is growing ", refers to the behaviour of *B. pyocyaneus* in these experiments, and is highly misleading. The " killing " referred to was presumably achieved, as in other experiments, at 45° C.—a temperature which is itself lethal for this organism. It is inconceivable that a concentration of any substance which is capable of killing an organism in aqueous suspension, would permit its growth when embodied in a culture medium, unless combination with some constituent of the medium rendered it inactive.

A further deduction is made by Churchman from his experiments with acid fuchsin. He observes (1923,*c*) that a mixture of two dyes with opposite selective actions should be a powerful therapeutic agent. Acriflavine having a selective action of the same type as acid fuchsin, and being miscible with gentian violet, he recommends a combination of these two agents for clinical use. It is this suggestion, which by implication credits " reversed bacteriostasis " with a general application in accordance with the Gram reaction, which may mislead a reader into supposing that a reversal of the violet dye type of action has been demonstrated. The selective action of these two agents is of the same type, both acting more weakly on Gram-negative than on Gram-positive bacteria ; their combination for clinical purposes has therefore not the theoretical advantage claimed for it.

CONCLUSIONS.

1. The survival of *B. anthracis* during exposure to acid fuchsin at 45° C. is shown to be dependent on the presence of spores. Vegetative *B. anthracis* and other Gram-positive bacteria are destroyed with greater rapidity by acid fuchsin and by acriflavine than are Gram-negative bacteria. The reversal of selective bactericidal effect in accordance with the Gram-staining reaction which this dye and other chemically related substances have been said to exhibit does therefore not exist.

2. Exposure to a temperature of 45° C., at which acid fuchsin has been said to exert this effect, is itself lethal for vegetative *B. anthracis* and for *B. pyocyaneus*. A demonstrable difference of only 2° C. separates the temperature at which *B. anthracis* will multiply and that at which it is destroyed.

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THE NITROGEN AND VITAMIN REQUIREMENTS OF *B. TYPHOSUS*.

PAUL FILDES,* G. P. GLADSTONE AND B. C. J. G. KNIGHT.*

From the Bacteriological Department, London Hospital.

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A STUDY of the food requirements of *B. typhosus* has been made by Van Loghem (1911) and Braun and Cahn-Bronner (1921, 1922). Taking the work of these latter authors as representing the present state of knowledge on nitrogen requirements, it appears that 18 out of 62 strains could grow aërobically when ammonia was the sole source of nitrogen. Growth was frequently delayed for several days or even weeks, but when once it started the culture could readily be propagated in the same medium. The remaining 44 strains neither responded to the offer of ammonia as the sole source of nitrogen, nor,

* Working with grants from the Medical Research Council.

indeed, of a number of amino acids. The authors, however, stated (1921) that growth of these latter strains could be obtained in the presence of 0·5 per cent. tryptophane, but later (1922) they expressed doubt of this result, their samples of tryptophane being small and impure. Still later (Braun, 1931) this effect was not referred to.

In the present paper two aspects of the growth requirements of *B. typhosus* are explored.

TECHNICAL DETAILS.

The general technique was as stated by Knight and Fildes (1933), and was designed to exclude from the cultures any unknown substance.

The *basal medium* consisted of the following mixture eventually brought up to 10 c.c. per tube with other constituents and water :

Sodium citrate 2·0 gm. ; $MgSO_4$ $7H_2O$, 0·5 gm. ; M/15 phosphate buffer, pH 7·4, 500 c.c., autoclaved.

0·5 c.c. of a 5 per cent. solution of glucose in water, autoclaved, was added per tube.

The *amino acids* used were alanine, glycine, leucine, valine, glutamic acid, asparagine, tyrosine, phenyl-alanine, proline, histidine, arginine, lysine, cystine and tryptophane. These were dissolved in water to 0·5 per cent., neutralized where necessary, autoclaved, and 0·1 c.c. added per tube. The following were, however, exceptions : Tyrosine was dissolved in N/10 NaOH ; cystine dihydrochloride was 0·2 per cent. in N/10 HCl, 1·0 c.c. being added per tube. In each case any necessary adjustments in pH were made in the medium. Tryptophane was made up to 0·08 per cent., and 0·25 c.c. added per tube.

Ammonia was added as ammonium chloride, 1·0 c.c. of an autoclaved 0·25 per cent. solution per tube.

The *strains* of *B. typhosus* used were (1) " Rawlings " (National Collection of Type Cultures No. 160), (2) " Malton " (N.C.T.C. No. 3758), (3) " Denby Dale " (N.C.T.C. No. 3759), (4) " Hersey " (N.C.T.C. No. 3760).

The *inocula* were of two varieties : (*a*) When judging the effect on growth of any constituent of the medium—for primary cultures a 24-hour growth on an agar slope was suspended in about 5 c.c. saline and 1 drop of this transferred to 10 c.c. saline ; of this 1 drop was inoculated. For subsequent cultures, 1 drop of one of the liquid cultures was diluted with 10 c.c. water and of this 1 drop was used. (*b*) When " training " 1 drop of the previous culture was used undiluted.

Purity of the reagents.—All reagents were of the finest commercial grade and were recrystallized 3 times. In the case of crucial materials further crystallizations were carried out—for instance, tryptophane 5 times.

Incubation.—All tubes were sloped in the incubator to allow greater aëration.

EXPERIMENTAL.

From Experiment 1 it is clear that all strains of *B. typhosus* tested were able to obtain their nitrogen readily from a mixture of amino acids when cultivated direct from agar.

TABLE I.

Experiment 1: *Cultivation of* 4 *Strains direct from Agar on Basal Medium, Glucose and* 14 *Amino Acids.*

Strain.	Growth (hours). Primary culture. 18.	Primary culture. 24.	1st subculture. 18.	2nd subculture. 18.
Rawlings	++	++	++	++
Malton	±	++	++	++
Denby Dale	+	++	++	++
Hersey	+	++	++	++

In this and other tables the *plus* signs are proportional to the opacity; tr. = trace.

The slight differences between the speeds of growth of the four strains were not constant. All strains could be subcultured indefinitely.

Determination of Essential Amino Acids.

A large number of experiments were carried out to determine which, if any, of the amino acids were essential for this growth. The method adopted was to omit one amino acid in turn from the mixture, or, in some cases, groups of amino acids which have been thought in animal physiology to be alternative; for instance, histidine and arginine. Experiment 2 shows the type of result obtained.

TABLE II.

Experiment 2: *Determination of Essential Amino Acids.*

Strain Rawlings: Inoculation from full amino acid mixture; basal medium, glucose and 14 amino acids.

	Growth (days). 2.	4.
14 amino acids	++	++
Lacking alanine	++	++
,, glycine	0	++
,, leucine	0	0
,, valine	+	++
,, glutamic acid	+	++
,, asparagine	++	++
,, tyrosine	0	++
,, phenyl-alanine	++	++
,, proline	++	++
,, histidine	0	++
,, arginine	++	++
,, lysine	0	0
,, cystine	±	+
,, tryptophane	tr.	tr.

From Experiment 2 it would appear that leucine, lysine and tryptophane were essential for growth, and that the absence of cystine in particular, and of glycine, valine, glutamic acid, tyrosine and histidine affected growth more or less adversely. This, however, was not found to be the case (Experiment 3).

TABLE III.

Experiment 3 : *Further Determination of Essential Amino Acids.*

Strain Rawlings ; inoculation from tube in Table II lacking glutamic acid ; nitrogen content made up with glycine ; basal medium, glucose and 9 amino acids.

	Growth (hours).		
	18.	36.	72.
9 amino acids	++	++	++
Lacking glycine	++	++	++
„ leucine	0	++	++
„ valine	++	++	++
„ glutamic acid	++	++	++
„ tyrosine	++	++	++
„ histidine	++	++	++
„ lysine	±	++	++
„ cystine	0	±	++
„ tryptophane	tr.	tr.	tr.

From Experiment 3 it is seen that leucine and lysine are no longer essential, but their absence merely causes delay. There is also delay in the absence of cystine, and no satisfactory growth in the absence of tryptophane.

Many other experiments were carried out which indicated that in the case of each of the four strains effective growth never occurred in the absence of tryptophane, while cystine seemed to exert a considerable influence. So far as the other amino acids were concerned, any difficulty which was caused by their absence could readily be overcome by " training ".

In the case of tryptophane a mere trace of growth is indicated in the protocols, but there seems to be little doubt that this is due to traces of tryptophane in the other amino acids and in the inoculum. Further purifications of the amino acids lead to a reduction in the frequency of this effect. It will be seen in Experiment 4 that the amount of tryptophane adequate to give a trace of growth is very small.

Experiment 4 shows that tryptophane will allow full growth when present in a concentration of 0·0004 per cent., and a trace when it is 125 times more dilute than this.

Elimination of Tryptophane.

Remembering that Braun and Cahn-Bronner were able to grow some strains of typhoid without great difficulty in the total absence of tryptophane, or indeed of all amino acids, we determined to test whether the process of training which could render the other amino acids superfluous would also operate in

TABLE IV.

Experiment 4 : *Determination of the Amount of Tryptophane necessary for Growth.*

Strain Rawlings ; inoculation direct from agar ; basal medium, glucose and 13 amino acids.

Tryptophane 0·25 c.c. of % solutions.	Growth.				
	Hours.			Days.	
	18.	24.	36.	4.	10.
0·08	⊥	++	++	++	++
0·016	⊥	++	++	++	++
0·0032	⊥	+	+	+	+
0·00064	tr.	⊥	+	+	+
0·00012	tr.	tr.	tr.	tr.	tr.
0·0	0	0	0	0	0

the case of tryptophane. The method of serial subculture in falling concentrations of tryptophane was used. We entirely failed with all four strains to obtain growths under the conditions found successful by Braun and Cahn-Bronner.

TABLE V.

Experiment 5 : *Elimination of Tryptophane.*

Strain Rawlings, already trained to grow on ammonium chloride and tryptophane ; basal medium, glucose, ammonium chloride ; inoculation from culture in ammonium chloride and tryptophane.

Tryptophane 0·25 c.c. of % solutions.	Growths (days).												
	¾.	1.	1¼.	10.		1.	4.	10.	1.	4.	¾.	1.	1¼.
0·08	⊥	+	+	++	→	0	0	0					
0·016	⊥	+	+	+	→	0	0	+	→ 0	0			
0·0032	⊥	⊥	+	+	→	0	0	0					
0·00064	⊥	⊥	⊥	⊥	→	0	0	+	→ 0	+	→ 0	tr.	+
0·000128	tr.	tr.	tr.	tr.	→	0	0	0		↘	0	tr.	+
0·0	tr.	tr.	tr.	tr.	→	0	0	0		↘	0	tr.	+

The arrows indicate subcultures. From the final tube containing no tryptophane, 12 serial subcultures have been made in ammonium chloride only.

Experiment 5 shows that in the presence of ammonium chloride tryptophane can be eliminated in three sub-cultures. The bacteria were then obtaining their nitrogen requirements from ammonia only.

This experiment was repeated with a Rawlings strain direct from agar. The tryptophane was eliminated first and the other amino acids afterwards. The strain Hersey also was trained to grow without tryptophane and other amino acids in three subcultures from agar, being then left with ammonia as the sole source of nitrogen.

Synthesis of Tryptophane.

As shown by Logie (1919–20), several members of the colon group are able to synthesize tryptophane when growing in a synthetic medium containing none. We have found this to be the case also with *B. aertrycke*. This organism was grown in a medium containing ammonium chloride and citrate as sole sources of nitrogen and carbon. The liquid culture diluted with an equal volume of alcohol was centrifuged, and the deposit from 250 c.c. added to a mixture of equal parts of glyoxylic acid reagent and concentrated sulphuric acid. In the control an equal volume of water was substituted for the glyoxylic reagent.

B. typhosus Rawlings trained to grow on a mixture of 13 amino acids without tryptophane and also when trained to grow on ammonia only when tested by this technique gave a positive result. It seems therefore clear that tryptophane is an essential feature of the make-up of this organism, that ordinarily it cannot synthesize it, and therefore it must be added to the medium. The process of training is clearly, at least in part, a training of the organism to synthesize tryptophane from other compounds containing nitrogen.

Effect of Sporogenes *Vitamin.*

It is already apparent that the vitamin-like substance which is essential for the growth of *B. sporogenes* (Knight and Fildes, 1933) need not be added to a medium in order to obtain growth of *B. typhosus*. If it is added to a medium containing 14 amino acids there is no acceleration. On the other hand, under some circumstances an effect may be detected :

TABLE VI.

Experiment 6 : *To Show the Effect of Adding* Sporogenes *Vitamin to the Medium.*

Basal medium, glucose, tryptophane, leucine, asparagine ; inoculation direct from agar.

Strain.	Growth (days). Without vitamin. 2.	3.	4.	With vitamin. 2.	3.	4.
Rawlings	0	±	+	0	tr.	+
Malton	0	+	+	0	+	+
Denby Dale	0	+	+	0	+	+
Hersey	0	0	+	0	+	+

Experiment 7.

As before, but tryptophane and glycine only.

Strain.	Growth (days). Without vitamin. 2.	3.	4.	With vitamin. 2.	3.	4.
Rawlings	0	0	0	0	0	0
Malton	0	0	0	0	0	+
Denby Dale	±	+	+	+	+	+
Hersey	0	0	0	0	0	0

Experiment 8.

As before, but asparagine, cystine, tryptophane.

Strain.	Growth (hours). Without vitamin. 15.	18.	72.	With vitamin. 15.	18.	72.
Rawlings	0	0	++	0	0	++
Malton	0	0	++	0	0	++
Denby Dale	0	0	0	0	0	++
Hersey	0	0	++	0	0	++

Experiment 9.

As before, but asparagine, leucine, cystine, lysine, tryptophane.

Strain.	Growth (hours). Without vitamin. 18.	24.	36.	48.	With vitamin. 18.	24.	36.	48.
Rawlings	0	0	+	+	±	±	+	+
Malton	0	0	+	+	tr.	tr.	+	+
Denby Dale	0	0	+	+	0	0	±	+
Hersey	0	0	+	+	tr.	tr.	+	+

The result of Experiments 6–9 may be summarized :

Strain.	Without vitamin.	With vitamin.
Rawlings improved	Once	Once
Malton ,,	0	Twice
Denby Dale ,,	Once	,,
Hersey ,,	0	,,

On two occasions the improvement consisted of growth with vitamin and no growth without, while the two instances of improvement without vitamin were extremely trivial.

The evidence thus seems to suggest that where inoculations are made direct from agar into a medium which is deficient in amino acids, the *sporogenes* vitamin may be shown to facilitate growth. It has been shown (Knight and Fildes, 1933) that the *sporogenes* vitamin is probably one of a closely related group of substances which have to do with the growth of bacteria, yeasts, moulds and plants. Some of these forms of life can synthesize the vitamin and others cannot. In the former case no vitamin need be added to the culture, in the latter it must be added. It may readily be shown (*cf.* Knight and Fildes, 1933) that *B. typhosus*, as would be expected, can synthesize this vitamin, both when growing on the full amino acid mixture and on ammonia. In fact one of the processes it must be trained to carry out when changing from agar through amino acids to ammonia is this vitamin synthesis. During this stage of training it is therefore not surprising that addition of vitamin will

produce a recognizable effect, as would the addition of one of the missing amino acids. The general effect of the evidence seems to support the suggestion that these organisms (*B. typhosus*, *B. aertrycke*, *B. tuberculosis*) which synthesize the vitamin do so because it is essential for their growth.

CONCLUSIONS.

1. *B. typhosus* can derive its nitrogen from a mixture of amino acids containing tryptophane, but ordinarily it will not grow without added tryptophane.
2. It can, however, be trained to grow without added tryptophane with ammonia as the sole source of nitrogen.
3. These trained organisms synthesize tryptophane when ammonia is the sole source of nitrogen.
4. It appears probable that tryptophane is an essential constituent of the protoplasm, and when the organism cannot synthesize it, it must be added to the medium.
5. Evidence is brought forward to suggest that *B. typhosus* requires for its growth the *sporogenes* vitamin.

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FILTRATION OF THE VIRUS OF BORNA DISEASE THROUGH GRADED COLLODION MEMBRANES.

W. J. ELFORD and I. A. GALLOWAY,

With an Appendix by J. E. Barnard.

From the National Institute for Medical Research, London.

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Borna disease, as is now well known, is an encephalo-myelitis, which may affect horses, cattle and sheep, and is transmissible by experimental inoculation to rabbits. Reference may be made to the following papers for full accounts of the experimental disease: Zwick, Seifried and Witte (1926), Nicolau and

Galloway (1927), Zwick, Seifried and Witte (1927), Nicolau and Galloway (1928, *a*), Galloway (1930), Nicolau and Galloway (1930, *a*); 1930, *b*).

Previous Work on the Filterability of the Virus.

The existing evidence on the filterability of the virus is very meagre. Zwick, Seifried and Witte (1926) made numerous filtration experiments, employing the various types of laboratory filter-candles (Berkefeld, V, N and W, and Pasteur Chamberland L1, L2, L3, L5 and L7). They also used Zsigmondy-Bachmann membranes. The material filtered was usually an emulsion of infective brain made in physiological saline, and subjected to a preliminary centrifugalization or filtration through a layer of asbestos wool to remove coarsely suspended matter. Apparently in two experiments only, out of the forty-four did these workers detect the presence of virus in bacteria-free filtrates. In one instance a 4 per cent. emulsion of brain in saline " cleared " by filtration through asbestos wool, furnished a filtrate containing only a small amount of virus when filtered through a Berkefeld N candle under a pressure of 2 atmospheres. On another occasion a similar emulsion yielded a filtrate containing a sufficient amount of virus to produce infection when filtered through a Zsigmondy-Bachmann membrane stated to be of pore size 0·75μ.

Broth was used as medium in one experiment only, but filtration of this emulsion through a Berkefeld N candle also yielded an inactive filtrate.

Nicolau and Galloway (1928, *a*) made two experiments with Mandler filter candles, the fluid filtered being an emulsion of infective rabbit brain in physiological saline. In one of these experiments the filtrate was without potency, while in the other the presence of a trace of virus was indicated by the death from the disease of one out of four rabbits inoculated.

These results show that although the infective agent of Borna disease may pass through filter-candles tested for their efficiency in retaining ordinary bacteria, yet it does so only with the greatest difficulty, and the concentration of virus in the filtrate is greatly reduced.

It was decided to investigate the filterability of the pathogenic agent of Borna disease, using the graded collodion membranes described by Elford (1931), and employing the technique of filtration analysis established by previous studies on viruses made in this Institute. (" Infectious Ectromelia," Barnard and Elford, 1931; " Foot and Mouth Disease," Galloway and Elford, 1931; " Vaccinia," Elford and Andrewes, 1932). The objectives of the present series of experiments have been to obtain fresh evidence on the filterability of the infective agent and to estimate the size of the virus particles.

EXPERIMENTAL.

Source of virus.—The tissue affinity of the virus of Borna disease is highly elective, all the available evidence indicating that nervous tissues alone contain the virus in high concentration. Infective brains of rabbits which were killed when showing very advanced symptoms of the disease have been used as the source of virus. Freshly prepared emulsions of brain in broth were always

employed, and brains stored in the cold have never served as the source of virus. The possibility of using diffusates was ruled out by the observation made by Nicolau and Galloway (1930, *a*) that the virus does not diffuse readily from brain-tissue into broth either at room temperature or at 37° C.

Preparation of stock virus emulsion.—A known weight of brain-tissue to give the required emulsion concentration (2 per cent. to 10 per cent.) was ground up with sterile quartz in Hartley's broth at pH 7·6, and then the whole was centrifugalized for 10 minutes at 2500 r.p.m., to sediment the coarse fraction. The supernatant liquid was filtered through a standardized sand and pulp filter (*vide* Barnard and Elford, 1931) to clarify it further of coarsely suspended material. The filtrate thus obtained was filtered through a permeable membrane of A.P.S. 0·75μ–0·85μ (the membranes used are graded in terms of the average pore size—A.P.S.) to furnish a bacteria-free stock filtrate of high virus potency. These stock filtrates were then examined further with less permeable membranes.

Method of testing for presence of virus.—0·25 c.c. of the liquid to be tested for the presence of virus was inoculated intracerebrally into healthy young Dutch rabbits. Two animals were used for each test, since considerable variation in susceptibility, especially to small doses of virus, is shown by rabbits. This procedure has been fully justified, for not infrequently one of the rabbits inoculated died of the disease, while the other one survived. Surviving animals were always tested for immunity.

Histological control.—In every case where an inoculated animal died after having shown typical symptoms, the brain was removed and fixed. Sections were examined for the characteristic infiltrative and degenerative lesions and for the presence of the Joest-Degen intranuclear bodies.

In some few cases in which the animals inoculated had shown signs of slight transient paresis during the period of observation, they were killed and an emulsion of the brain passaged by intra-cerebral inoculation into fresh rabbits, but in no instance was any evidence of the presence of virus obtained. Rabbits which had not shown any signs of the disease during the period of the experiment were re-inoculated with 0·25 c.c. of a 10 per cent. infective brain emulsion, but no case of immunity was recorded.

Strain of virus.—An equine strain of the virus which was kindly sent by Prof. Zwick of Giessen in 1926 has been used in these experiments. This strain has been passaged many times through rabbits, and the slight modifications it has undergone in respect of the period of incubation, and the number, size and localization of the Joest-Degen intranuclear bodies, have already been described by Nicolau and Galloway (1931).

The rate of progress in the experimental study of the disease is rendered very slow owing to the exceptionally long incubation period. In rabbits inoculated intra-cerebrally with 0·25 c.c. of a 10 per cent. centrifugalized brain emulsion, the incubation period has never been less than four weeks. With high dilutions of virus the incubation period is generally longer. Thus rabbits inoculated with 0·25 c.c. of a 1/5000 to 1/10,000 dilution of centrifugalized emulsion have developed symptoms, on an average, in 40 days, and have died or been killed *in extremis* in between 45 to 55 days. As a general rule, when the incubation period is prolonged it may be assumed that the amount of virus

contained in the inoculum has been small. The rabbits in the series of experiments here described have been kept under observation for 100 days, and where in the tables it is recorded that the animal survived (S.), this means that it survived for that period without having shown any symptoms of disease. It is fully realized that even longer incubation periods than 100 days have been observed, but such instances are exceedingly rare.

Filtration technique.—The technique of ultra-filtration in these experiments has been precisely that employed in previous analyses of virus suspensions, to which reference has already been made. The graded collodion membranes described by Elford (1931) have been used, and the filtration operation carried out under positive applied pressures of air or nitrogen.

RESULTS OF FILTRATION EXPERIMENTS.

Preliminary experiments were made with centrifugalized emulsions of brain in physiological saline and also in broth (15 min. at 2500 r.p.m.). The supernatant liquid was very turbid, and quickly blocked even the most permeable membranes. As will be seen from Table I, a 0·6μ membrane gave an inactive filtrate with the saline emulsion, but when a broth emulsion was filtered, an active filtrate was obtained. These experiments, therefore, showed that further clearing was essential as a preliminary to membrane filtration, and also that broth was the better medium to use.

In subsequent experiments the centrifugalized emulsion was filtered through a sand and pulp filter, standardized by the method described in a previous paper (Barnard and Elford, 1931). The filtrate thus obtained was usually slightly opalescent in appearance. Table I contains data of experiments in which such sand and pulp filtrates were filtered through progressively finer grades of membranes. The 0·5μ membrane filtrate showed little loss of infectivity compared with the original unfiltered stock liquid but with the 0·3μ membrane there was a very noticeable drop in the infectivity of the filtrate.

The procedure already described (*vide* p. 198) finally adopted for preparing the stock virus emulsion has furnished stock filtrates possessing consistently high virus activity. It will be seen from Table II that the limiting dilution was usually 1 : 10,000 at least, and sometimes slightly higher. In addition to these stock filtrates used for the filtration studies, seven filtrates were prepared in the same way for the studies described in the appendix. Although no estimation of the virus content of these filtrates by accurate titration methods was made, the fact that all the inoculated animals died or were killed in an advanced stage of the disease in from 32 to 55 days (an average of 45 days for 14 rabbits) showed that they were of high virus potency. The consistency with which such stock filtrates have been obtained is in itself a great advance upon the results reported by previous workers, whose experiments with candles were conspicuous in their regularity in yielding inactive filtrates, or filtrates of very low potency. The success of the present method may be ascribed to three factors : (1) The recognition of the *rôle* played by the medium in filtration. It will be observed that broth has been used, whereas physiological saline had been favoured by previous workers. (2) The gradual fractionation of the

emulsion of brain-tissue, for which purpose centrifugalization, the use of standardized sand and pulp filters and graded collodion membranes have been combined. This systematic treatment contrasts with the procedure formerly adopted, in which the centrifugalized emulsion was usually filtered directly through a filter candle, which naturally quickly blocked. (3) The availability of a graded series of membranes of reproducible quality.

The Filtration End-point and Probable Size of the Virus.

Table III contains a summary of the results obtained on filtering the stock filtrates through membranes of progressively finer grade. The first noticeable drop in the virus concentration of the filtrate as compared with that of the original stock filtrate occurs at about 0·4μ; the falling off continues rapidly to 0·2μ. In the region of 0·2μ the results have been variable, sometimes a positive filtrate being obtained and sometimes a negative, according to the prevailing conditions—concentration of virus in stock filtrate, volume filtered and applied pressure. The absolute end-point was determined to be 0·175μ, from which the probable size of the smallest virus units is estimated to be 0·085μ–0·125μ (Elford, 1933). However, the end-point is not as sharp as that obtained with some other viruses, for example, that of infectious ectromelia of mice. This would suggest that the virus particles of Borna disease may not be quite so uniformly dispersed, or exhibit the same uniformity in individual size. There would appear to be little difference in the average size between the virus of Borna disease and that of infectious ectromelia, since the main change of slope in the filtration curves occurs at an almost identical zone of permeability; yet the fact that a 0·2μ membrane consistently gave an inactive filtrate with the virus of infectious ectromelia, but has occasionally, when conditions have been favourable, given positive filtrates with the virus of Borna disease, would indicate that the smallest phase of the latter virus is somewhat smaller than that of infectious ectromelia of mice.

Influence of Hydrogen Ion Concentration upon the Stability and Filterability of the Virus.

In varying the pH value of broth medium N/10 hydrochloric acid and sodium hydroxide were used. The use of phosphate buffers was not practicable, since the high concentration M/15 of phosphate salts frequently proves fatal to rabbits when inoculated intracerebally in doses of 0·25 c.c. The actual pH values of the systems were always checked electro-metrically.

The virus appears to be very sensitive to changes in the pH value of the medium at room temperature. Thus a broth suspension of the virus, which after 3 hours at pH 7·6 was infective in 1/5000 dilution (highest dilution tested), had its infectivity reduced in 1 hour at pH 8·5 to such an extent that a 1/50 dilution was non-infective. The corresponding suspension at pH 5·68 after 1¼ hours had also deteriorated, but in less marked degree, the 1/5000 dilution now being non-infective, while the 1/50 dilution remained potent. Before inoculation the pH values of acid and alkaline filtrates were always readjusted to

neutrality (pH 7·4–7·6). These observations are in agreement with the findings of Nicolau and Galloway (1928, *b*; 1930, *a*), namely, that the virus is more sensitive to the action of alkali than to acid. Clearly no reliable filtration experiment could be made at a pH as high as 8·0. Table IV contains the results of some filtration experiments at slightly acid pH values. The virus suspensions were prepared by adding 5 c.c. of stock filtrate pH 7·6 to each of three 25 c.c. volumes of broth which had been acidified by adding N/10 HCl. This ensured that each system contained initially the same amount of virus. As seen in the table, the only positive filtrate with 0·3μ membranes was given by the system at pH 7·5. It is evident from the control tests that even at pH 6·5 the potency of the suspension falls off appreciably during the period necessary for the experiment—6 hours approximately. In view of this fact, further experiments were not made. The evidence obtained was sufficient to show that our standard condition of filtration at pH 7·4–7·6 was the optimum both as regards the stability of the virus and its filterability.

Ultra-filtration as a Means of Concentrating the Virus.

The end-point in membrane permeability for the virus having been determined as 0·175μ, it was reasonable to expect that some concentration of the virus might be effected with a 0·15μ membrane. Two experiments, the data for which are given in Table V, showed that this was so. The small volume of residual fluid remaining in the filter was found to possess greater potency than the original suspension. It will be seen from the table that the actual concentration was at least five-fold, whereas if all the virus present in the original volume were assumed to be retained above the membrane, one would anticipate the concentration in the residual volume to be nearly ten-fold the original. However, some virus will most certainly adhere to the membrane, and it is possible also that some aggregation of virus particles may occur. These factors would serve to explain the somewhat lower infectivity of the residue.

CENTRIFUGALIZING EXPERIMENTS.

Three experiments in centrifugalizing stock membrane filtrates of the virus for 6 hours at 3000–3500 r.p.m. were made. The results obtained were very comparable in all three cases. Table VI gives the protocol of one of these experiments. There was a slight sediment, which was shaken up with the 0·5 c.c. fluid tested as the bottom sample. A slight but definite enhancement of the infectivity of the bottom sample was found, but it was also very evident from the tests made, that the bulk of the virus particles remained suspended, for even the uppermost sample had diminished but little in its potency. The sedimented fraction probably consisted of the largest particles—maybe protein-virus complex or virus aggregates. The results demonstrate the importance of measuring the concentration of the dispersed virus at different parts of the centrifuge tube after the experiment, and not having concern solely for the sediment. In order to analyse correctly the experimental results, it is necessary to have a balance and loss account for representative samples of the complete system.

TABLE I.—*Preliminary Filtration Experiments.*

Stock fluid.	Experiment No.	Highest infective dilution of stock fluid and time of death in days.	Medium.	Membrane grade. (μ.)	Filtration pressure. (cm. Hg.)	Volume of filtrate. (c.c.)	Infectivity of filtrate. Filtrate dilution.	Infectivity of filtrate. Time of death in days.	Remarks.
Supernatant liquid from 10% centrifugalized brain emulsion	1	1 : 5000*; 35	Broth	0·87	76	5	1 : 10,000	46	The membrane rapidly became blocked owing to the retention at the membrane surface of coarsely dispersed material.
	2	1 : 10,000*; 42	,,	0·85	20	3	1 : 5000	39 –	
				0·60	20	2	Undil.	39 ; 65	
	3	1 : 10,000*; 45	Saline	0·85	20	3	1 : 5000	45	
				0·60	20	2	Undil.	S ; S	
Filtrate from a sand and paper pulp filter, made from the supernatant fluid of a 10% centrifugalized brain emulsion	4	1 : 5000; 35 / 1 : 20,000; S.	Broth	0·50	76	10	1 : 10,000	46	
				0·40	20	6	Undil.	39, 29, 35, 46	
				0·26	20	6	,,	S, S, S, S	
	5	1 : 5000; 45	Broth	0·30	76	(i) 5	,,	S. S	Three successive samples of filtrate were taken.
						(ii) 5	,,	65, S	
						(iii) 10	,,	38, S	

* = No higher dilution tested. S = Survived.

TABLE III.—*Filtration of the Virus through Membranes of Progressively Finer Pore Size.*

Brain emulsion. (%.)	Infectivity of stock filtrate. Highest active dilution.	Infectivity of stock filtrate. Time of death in days.	A.P.S. of membrane in μ.	Membrane thickness. (m.m.)	Filtration pressure atmosphere.	Infectivity of filtrate. Sample 1. Volume. (c.c.)	Sample 1. Virus test.	Sample 1. Time of death in days.	Sample 2. Volume. (c.c.)	Sample 2. Virus test.	Sample 2. Time of death in days.	Sample 3. Volume. (c.c.)	Sample 3. Virus test.	Sample 3. Time of death in days.
10	1 : 5000	35	0·500	0·180	1	10	+	46	..	..	..	..	..	..
			0·400	0·170	1	6	+, +	39, 35	..	..	..	..	..	..
10	1 : 5000	45	0·300	0·180	1	5	0, 0	S, S	5	+, 0	65, S	10	+, 0	38, S
2	1 : 10,000	32	0·300	0·180	1	5	+, 0	39, S	..	..	..	..	..	..
10	1 : 5000	47	0·260	0·150	2	10	+, +	56, 41	5	+, +	48, 54	7	+, +	54, 84
2	1 : 10,000	83	0·250	0·160	2	5	+, +	42, 47	5	+, +	37, 42	..	..	..
			0·250	0·160	1	5	0, 0	S, S	5	+, 0	42, S	..	..	..
10	1 : 10,000	51	0·210	0·120	2	5	0, 0	S, S	5	0, 0	S, S	5	0, 0	S, S
5	1 : 10,000	49	0·220	0·120	1	6	+	65	5	+, +	49, 49	..	..	..
2	1 : 10,000	96	0·220	0·120	2	5	0, 0	S, S	5	+, 0	51, S	10	+, 0	51, S
2	1 : 5000	30	0·200	0·150	1	5	0, 0	S, S	5	0, 0	S, S	5	0, 0	S, S
5	1 : 5000	55	0·200	0·125	2	5	0, 0	S, S	5	+, +	43, 48	5	+, 0	49, S
			0·200	0·125	2	5	0, 0	S, S	5	+, 0	49, S	..	..	..
2	1 : 10,000	83	0·175	0·120	2	5	0, 0	S, S	5	0, 0	S, S	..	..	..
			0·175	0·120	1	5	0, 0	S, S	5	0, 0	S, S	..	..	..
5	1 : 5000	58	0·150	0·120	2	5	0, 0	S, S	5	0, 0	S, S	5	0, 0	S, S
			0·150	0·120	1	5	0, 0	S, S	5	0, 0	S, S	..	..	..
5	1 : 10,000	48	0·150	0·120	2	7·5	0, 0	S, S	5	0, 0	S, S	..	..	..
10	1 : 5000	47	0·125	0·125	2	10	0, 0	S, S	..	..	..	..	..	..

+ = Virus present ; 0 = virus absent ; S = survived. In all filtrate tests the undiluted filtrate was used.

TABLE II.—*Titres of Stock Filtrates.*

Concentration of brain emulsion. (%.)	Membrane grade. (μ.)	Filtration pressure. (cm. Hg.)	Titre of filtrate.	Time of death (in days) after inoculation with highest active dilution.
10	1·00	76	>1 : 5000 ; <1 : 25,000	47 ; 48
10	1·00	76	1 : 10,000*	51
10	0·60	76	1 : 10,000*	51
5	0·75	20	>1 : 10,000 ; <1 : 50,000	49
5	0·75	20	>1 : 10,000 ; <1 : 50,000	48
5	0·65	76	>1 : 10,000 ; <1 : 100,000	40 ; 61
5	0·65	76	>1 : 5000 ; <1 : 20,000	55
2	1·00	76	1 : 10,000*	32
2	0·80	76	1 : 10,000*	49
2	0·75	76	>1 : 10,000 ; <1 : 50,000	76
2	0·65	76	>1 : 5000 ; <1 : 20,000	30
2	0·60	76	1 : 10,000*	96

In "titre of filtrate" column : > the highest active dilution ; < an inactive dilution.

SEPARATION OF THE VIRUSES OF LOUPING-ILL OF SHEEP AND BORNA DISEASE.

Simultaneously with the experiments with the virus of Borna disease a study was being made of the filterability of the virus of "louping ill" of sheep. The end-point in membrane permeability for the latter virus was found to be separated from that of the virus of Borna disease by more than 100μμ, so that little difficulty would be anticipated in effecting a resolution of a mixture of these two viruses by means of filtration. Separation was shown to be readily possible by the following experiment :

A 10 per cent. emulsion in broth of infected mouse brain was centrifuged, and the supernatant liquid filtered through a sand and pulp filter. The filtrate thus obtained was in turn filtered through a 0·5μ membrane to yield a stock filtrate of louping-ill virus. At the same time a stock filtrate of the virus of Borna disease was prepared in the usual manner from a 10 per cent. broth emulsion of infected rabbit brain, using a 1·0μ membrane. Both stock filtrates were kept in the cold (3° C.) overnight. A mixture of equal volumes of the filtrates of the two viruses was made, and after thorough shaking to ensure proper mixing, this was filtered through a membrane of grade 0·125μ, *i. e.* intermediate between the end-points of the viruses. Filtration was conducted at 76 cm. mercury, and 7·5 c.c. were filtered through 1 sq. cm. membrane. The filtrate when tested was found to contain the virus of louping ill of sheep but not that of Borna disease. This was in accordance with the anticipated result, the virus of the former disease being definitely the smaller of the two.

SUMMARY.

1. A technique for the regular production of bacteria-free and highly active filtrates of the virus of Borna disease from emulsions in broth of infective rabbit brain is described.

TABLE IV.—*Effect of pH upon the Filterability of the Virus.*

pH of system.	Control test. System at room temperature for 6 hours. Dilution.	Test.	Time of death (days).	Test on filtrate (undiluted). Test.	Time of death (days).	Remarks.
7·5	1 : 10,000	+, +	32, 32	+, 0	39, S	Interval between time of preparation of 10% stock emulsion and the inoculation operation approximately 6 hours. Filtration conditions in each case: Membrane, 0·3μ; pressure, 76 cm. Hg.; volume filtered, 12·5 c.c.
6·47	1 : 10,000	+, 0	39, S	0, 0	S, S	
5·6	1 : 10	+, +	39, 69	0, 0	S, S	
	1 : 10,000	0, 0	S, S	..	..	

TABLE V.—*Concentration of the Virus above an Impermeable Membrane.*

Experiment.	Infectivity of stock fluid. Dilution.	Test.	Time of death (days).	Membrane.	Charge in filter. (c.c.)	Volume of filtrate. (c.c.)	Volume of residue. (c.c.)	Infectivity of filtrate.	Infectivity of residue. Dilution.	Test.	Time of death (days).
1	1 : 10,000	+	48	A.P.S. 0·15μ thickness 0·12 mm., area 1·05 sq. cm.	15	13·5	1·5	Inactive	1 : 50,000	+	45
	1 : 50,000	0	S						1 : 100,000	0	S
									1 : 200,000	0	S
2	1 : 5,000	+	43	A.P.S. 0·15μ thickness 0·12 mm. area 2·5 sq. cm.	20	18	2	,,	1 : 10,000	+	33
	1 : 10,000	+	49						1 : 50,000	+	51
	1 : 50,000	0	S						1 : 100,000	0	S

TABLE VI.—*Protocol of a Centrifugalization Experiment.*

Infectivity of stock filtrate. Dilution.	Test.	Time of death (days).	Centrifuging conditions.	Uppermost layer. Dilution.	Test.	Time of death (days).	Middle fraction. Dilution.	Test.	Time of death (days).	Bottom sample. Dilution.	Test.	Time of death (days).
1 : 5000	+	43	6 hours at 3500 r.p.m.	1 : 5000	+	48	1 : 5000	+	48	1 : 10,000	+	48
1 : 10,000	+	49		1 : 10,000	+	72	1 : 10,000	+	65	1 : 50,000	+, 0	48, S
1 : 50,000	0	S		..	..	..	1 : 50,000	0	S	1 : 100,000	0, 0	S, S

Remarks: The centrifuge tube (length 11·5 cm., diameter 1·25 cm., and tapering at bottom) was calibrated to enable an uppermost sample 1 c.c., a middle sample 2 c.c., and a bottom sample 0·5 c.c. to be withdrawn for test. There was a visible deposit in tube. This was shaken up in the bottom 0·5 c.c. sample.

Stock filtrate used: 0·75μ filtrate of 5% brain emulsion in broth.

2. The size of the smallest phase of the virus of Borna disease has been estimated to be 0·085μ–0·125μ from the results of experiments on its filterability through graded collodion membranes.

3. The optimum hydrogen ion concentration for maximum stability of the virus in broth medium at room temperature, 15–20° C., corresponds to pH 7·4–7·6. The virus is very sensitive to changes of pH outside this zone, particularly on the alkaline side.

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APPENDIX.

By J. E. BARNARD.

The filtration results obtained by Elford and Galloway suggest that the virus of this disease is particulate, and that the size of the organism is comparable to that of infectious ectromelia or vaccinia. It should therefore be possible to obtain dark-ground ultra-violet photo-micrographs of such an organism; and attempts have been made with brain emulsions and filtrates known to be infective. It is not claimed that the results are final or conclusive, but they are at least sufficiently suggestive to place on record. The animal experiments and the filtration results have in all cases been in close relation with the microscopical findings, the presence or absence of granular bodies of a certain size and of particular optical properties being significant. Seven highly infective membrane filtrates were examined and virus particles were found in each. It is not proposed in this communication to set out particular experiments in detail; it must suffice to refer to the general agreement in the results. The work is proceeding, and it is expected that further evidence in support of the present suggestions will be obtained. Actually it has been found difficult to dissociate the virus bodies from emulsions of brain tissue. Apparently this arises from two causes: the organism is widely distributed throughout the brain, but does not occur in such great quantity as it does in the epithelial tissues of the mouse in infectious ectromelia or in the canary in canary pox; further, it remains more closely associated with the cellular elements in the brain even in a rabbit dead of the disease, with the result that preliminary breaking down of the tissue to obtain an emulsion

must be carried out more completely. From a microscopical standpoint this increases the difficulty of observation, as many more particles of tissue *débris* of similar size to the virus remain in any filtrate, and must be differentiated from the virus bodies. Thus the almost total replacement of normal cell structures, as in infectious ectromelia, by masses of virus bodies apparently does not occur, so that the dissociation of virus from cells is more difficult. Recent improvements in technique suggest that these difficulties will be overcome, and further experiments now in progress are encouraging. The procedure has been similar to that adopted in the identification of the virus of infectious ectromelia (Barnard and Elford, 1931). Infective filtrates are examined in visible light, and bodies of fairly constant size, but more particularly with constant optical properties, can be identified, latterly with increasing certainty, as experience has been gained. Photo-micrographs in ultra-violet light, by a method capable of resolving bodies of the expected size, are then obtained, some examples of which are shown.

DESCRIPTION OF PLATE.

FIG. 1.—*Chr. prodigiosum.* × 3200. A standard of reference for estimation of relative size. All these photographs are on exactly the same scale.

FIG. 2.—The virus bodies identified in canary pox.

FIG. 3.—Vaccinia virus, testicular strain.

FIG. 4.—Borna disease virus bodies from membrane filtrate, emulsion in broth. About 10 c.c. of filtrate was centrifuged until a visible deposit was obtained in a narrow tube. The sediment was removed and mixed with about 1 c.c. of Ringer's solution to reduce the broth content to a minimum. This procedure makes it easier to obtain photo-micrographs in ultra-violet light, as broth is less transparent to the wave-length used than is Ringer's solution.

FIG. 5.—Borna disease virus bodies from sand and paper pulp filtrate. The largest body in this field is a sand grain, not virus.

FIG. 6.—A sand and paper pulp filtrate was centrifuged for 1 hour at 3500 r.p.m. The upper part of the supernatant fluid was removed and again centrifuged for 4 hours at the same speed. Characteristic bodies could be found in small numbers, usually one or two per field. Centrifugates obtained by such means are infective. The size of the virus bodies in this disease appears to vary within wider limits than in any other virus hitherto examined by the same methods. Filtration results also suggest that there is such a variation. The photographs suggest a size varying from 0·11μ to 0·14μ.

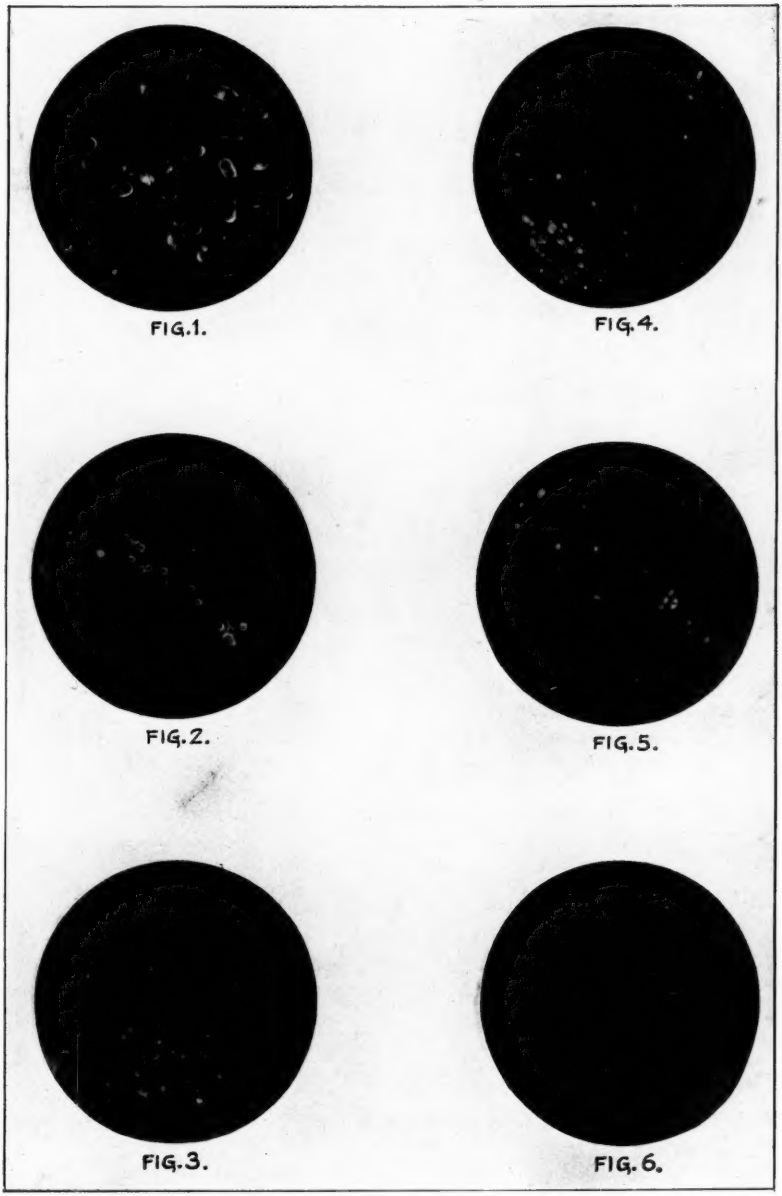

FIG. 1.

FIG. 4.

FIG. 2.

FIG. 5.

FIG. 3.

FIG. 6.

Barnard.